An Investigation of the pharmacological modulation of Diffuse Noxious Inhibitory Controls in a Monoiodoacetate model of Osteoarthritis

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I, Stevie Margaret Lockwood, confirm that the work presented in this thesis is my own. Where information has been delivered from other sources, I confirm that this has been indicated in the thesis.

Abstract

Osteoarthritis (OA) is a disabling disease of the synovial joints. Structurally, OA involves the degenerative breakdown of articular cartilage, inflammation of the synovium and sclerosis of the subchondral bone, which ultimately results in a loss of function and integrity of the synovial joints. Clinically, it is the chronic pain associated with OA that causes patients to seek medical attention. Traditional analgesics used to relieve the chronic pain associated with OA aim to alleviate peripheral nociception at the level of the diseased joint. However, OA patients often develop referred pain and suffer with chronic pain even after total joint replacement surgery, which indicates that the chronic pain associated with OA is not always driven by purely peripheral mechanisms. Therefore, a better understanding of the central mechanisms involved in the development of OA associated chronic pain may aid the development of effective analgesics that tackle the centrally driven element.

Diffuse Noxious Inhibitory Controls (DNIC) describes the phenomenon where one pain inhibits another; this system utilizes endogenous descending inhibitory controls, which mediate an inhibitory effect at the level of the spinal cord. In order to characterize this system in rats, *in vivo* electrophysiological recordings were made from single unit dorsal horn convergent neurons. The dorsal horn neurons were activated by mechanical and thermal stimulation of the hind paw and DNIC was induced by a concurrent noxious conditioning pinch. This thesis aimed to investigate the pharmacology of DNIC.

OA of the knee was modeled in this thesis with a 2mg monoiodoacetate (MIA) intrarticular injection in the rat. This thesis investigated central neuronal plasticity with spinal electrophysiology, assessed the functionality of descending controls through measuring DNIC responses, and characterized the joint histopathology and pain-like behaviour associated with this model.

Studies presented in this thesis confirmed that DNIC are mediated via noradrenergic inhibitory descending controls, but that serotonergic descending controls can also influence the expression of DNIC. Early phase MIA animals (2-6 days post injection) displayed significant mechanical hypersensitivity, yet showed little articular cartilage degradation and had a functional DNIC system. DNIC expression was abolished in early phase animals through blocking the actions of spinal α_2-adrenoceptors with the antagonist atipamezole, while blocking the actions of spinal 5-HT_7 receptors reduced DNIC expression. Late phase MIA animals (14-20 days post injection) demonstrated significant articular cartilage degradation, mechanical hypersensitivity, and an abolished DNIC system. DNIC expression was restored in late phase MIA animals by enhancing noradrenergic modulation with the NRI and μ-opioid receptor agonist tapentadol, and by activating spinal 5-HT_7 receptors with the selective 5-HT_7 receptor agonist AS-19. In addition, pregabalin significantly reduced spinal neuronal responses in late phase MIA animals yet had no effect in early phase animals. Overall, these results suggest there is a decreased noradrenergic inhibitory descending drive acting at spinal α_2-adrenoceptors, and modulation of serotonergic descending controls acting at spinal 5-HT_7 receptors in late phase MIA animals.

The studies presented in this thesis demonstrate the applications of assessing DNIC in this experimental model of OA, that the DNIC system becomes abolished as the model progresses, and that DNIC can be restored through pharmacologically manipulating monoaminergic descending controls. Overall, this may represent a strategy for relieving centrally driven OA associated chronic pain.

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Declaration of published work

Some of the work presented in this thesis has been previously presented or published.

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Abbreviations

5-HT – serotonin
5-HTT – serotonin transporter
ADAMTS - A distintegrin and metalloproteinase with thrombospondin motifs
AMPA - α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid
ANOVA – analysis of variance
ATP – adenosine triphosphate
BDNF – brain-derived neurotrophic factor
Ca^{2+} - calcium
CaMKII - Ca^{2+}-calmodulin-dependent protein kinase II
cAMP – cyclic Adenosis Monophosphate
CatK – Cathepsin K
CatS – Cathepsin S
CC – cingulate cortex
CFA – complete Freunds adjuvant
CGRP – calcitonin gene-related peptide
Cox – cyclooxygenase
CPM – Conditioned Pain Modulation
CREB – cAMP response element binding protein
CVLM - caudal ventrolateral medulla
dlPFC – dorsolateral Prefrontal Cortex
DNIC – diffuse noxious inhibitory controls
DRG – dorsal root ganglia
DTT – dithiothreitol
ECM – extracellular matrix
EPAC – cAMP activated guanine exchange factor
FKN – Fractalkine
fMRI – functional magnetic resonance imaging
GAPDH - Glyceraldehyde 3-phosphate dehydrogenase
GFAP - glial fibrillary acid protein
GM-CSF – granulocyte-macrophage colony stimulating factor
GP – General practitioner
GPCR – G-protein coupled receptors
HA – hyaluronic acid
IASP – International association for the study of Pain
Iba1 - Ionized calcium binding adaptor molecule 1
IBS – Irritable bowel syndrome
IL-10 – Interleukin 10
IL-17 – Interleukin 17
IL-1β – Interleukin 1 beta
IL-6 – Interleukin 6
IL-8 – Interleukin 8
IL1-R1 – Interleukin 1 Receptor Type 1
IMS – Industrial Methylated Spirits
IP_3 - Inositol 1,4,5-triphosphate
K^+ - potassium
Lab – laboratory
LC – locus coeruleus
LPb - lateral parabrachial area
LTP – long term potentiation
MAPK – mitogen-activated protein kinase
MIA – monoiodoacetate
MMP – Matrixmetalloproteinases

NA – noradrenaline
Na^+ - sodium
NFκB – Nuclear Factor κB
NGF – nerve growth factor
NHS – National health service
NK-1 – neurokinin 1
NMDA - N-methyl D-aspartate
NRI – noradrenaline reuptake inhibitor
NRI – noradrenaline reuptake inhibitor
NRI – noradrenaline reuptake inhibitor
NS – nociceptive specific
NSAIDs – non-steroidal anti-inflammatory drugs
NTS - nucleus of the solitary tract
OA – osteoarthritis
PAG – periaqueductal grey
PbN – parabrachial nucleus
pCPA - p-chlorophenylalanine
PD – Post Discharge
PFA – paraformaldehyde
PGE2 – Prostaglandin E2.
PKA – Protein kinase A
PKC – Protein kinase C
PPT – pressure pain threshold
PSTH – Post stimulus time histogram
PWT – paw withdrawal threshold
PWT – paw withdrawal threshold
qPCR – quantitative polymerase chain reaction
QST – quantitative sensory testing
QST – quantitiative sensory testing
RA – rheumatoid arthritis
ROS – Reactive oxygen species
RVM – rostroventral medial medulla
SEM – Standard error of the mean
SEM – standard error of the mean
SNL – spinal nerve ligation
SNRI – serotonin noradrenaline reuptake inhibitor
SNRI – serotonin reuptake inhibitor
SRD – subnucleus reticularis dorsalis
SSRI – selective serotonin reuptake inhibitor
TIMP – Tissue inhibitor of metalloproteinase
TMD – transmembrane domain
TNFR1 – Tumor Necrosis Factor Receptor 1
TNFα – Tumor Necrosis Factor alpha
TrkA – tyrosine kinase A
TrkB – tyrosine kinase B
TRP – transient receptor potential
VGCC – voltage gated calcium channels
WDR – Wide dynamic range
WHO – World health organization

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1. Introduction

1.1 The nociceptive system

1.1.1 What is pain?

The International Association for the Study of Pain describes pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Merksey and Bogduk 1994). Pain is a warning mechanism that exists to prevent tissue damage, however the experience of pain can often outlast its protective purpose and remain present for weeks, months and even years. Nociception is the process in which the sensory nervous system transmits information and responds to potentially harmful stimuli, while pain is a highly subjective experience influenced by memories, emotional, genetic and cognitive factors and the resultant perceived pain is not always be equal to the stimulus (Tracey and Mantyh 2007).

Conceptually, pain can be classified according to its persistency. Acute pain is commonly associated with ongoing tissue damage and but is time limited, in contrast chronic pain persists for longer periods of time exceeding 3 months outlasting its biological efficacy (Merskey and Bogduk 1994). If the ongoing tissue damage does not recover acute pain can become chronic and outlast the usual time of healing, therefore it is important to treat acute pain before it can progress to this more severe, chronic pain state (Lynch 2011). Both acute and chronic pain are an enormous social and economic burden severely reducing a patients quality of life, and the primary management of chronic pain accounts for 4.6 million general practitioner (GP) appointments a year in the UK costing the NHS 69 million pounds (Phillips 2009). There is an unmet need for treatment and management of chronic pain, thus understanding the biological mechanisms underlying pain processing and its circuitry is crucial for the development of therapeutic targets.

The basics of pain circuitry have been established. The pain signal is initiated in the peripheral afferent fibres, which carry nociceptive stimuli as electrophysiological activity to superficial spinal dorsal horn neurones. Within the dorsal horn, nociceptive information is then modulated and transmitted along ascending pathways to higher centres in the brain via projection neurones (Porecca 2002). The main termination sites for the ascending projections are the brainstem and thalamus, which contain higher

order neurones that project the signal to various cortical regions, which subsequently modulate the different aspects of the pain experience (Fields 2004). These supraspinal sites integrate a response and activate the relay of descending pathways to modulate the excitability of deeper dorsal horn neurones and ultimately determine the perception of pain (Bridgestock and Rae 2013).

Pain can be classified into broad categories based on the underlying mechanism (Figure 1.1). Firstly, there is nociceptive acute pain; this is an early warning physiological protective system essential for sustaining bodily integrity. This is the type of pain felt when stubbing ones toe or touching something too hot, cold or sharp. The withdrawal reflex activated by this high threshold pain causes immediate attention and withdrawal from the intense noxious stimuli (Woolf 2010). Neuropathic pain is a type of chronic pain caused by a lesion or disease state affecting the somatosensory system (Treede et al 2008)(Backonja 2003). Post herpetic neuralgia (PHN), diabetic neuropathy and human immunodeficiency virus (HIV) related neuropathies are all examples of damage to the periphery, subsequently transmission of signals from the afferent to the nervous system is partially or completely lost (Jenson and Baron 2003). Nonetheless, neuropathic pain can also be caused by lesion to the CNS, which occurs in multiple sclerosis, stroke and spinal cord injury. A second mechanism underlying the development of chronic pain is inflammation, whereby damaged tissues release chemical mediators that can activate and sensitise the pain signaling system. Many conditions cause this sort of pain including osteoarthritis (OA), rheumatoid arthritis (RA) and post-operative pain. Neuropathic and inflammatory pain can also occur at the same time, a good example is the pain related to cancer; as the tumour expands it causes tissue damage and the release of inflammatory mediators as well as causing nerve damage through compression and invasion. Recently, evidence has suggested it also has unique features and so should be considered a separate pain state (Falk and Dickenson 2014). Finally, in generalized or dysfunctional pain there is no obvious damage or inflammation to the nervous system yet patients feel substantial pain. Conditions evoking dysfunctional pain include fibromyalgia, irritable bowel syndrome (IBS), tension headaches and interstitial cystitis (Woolf 2010).

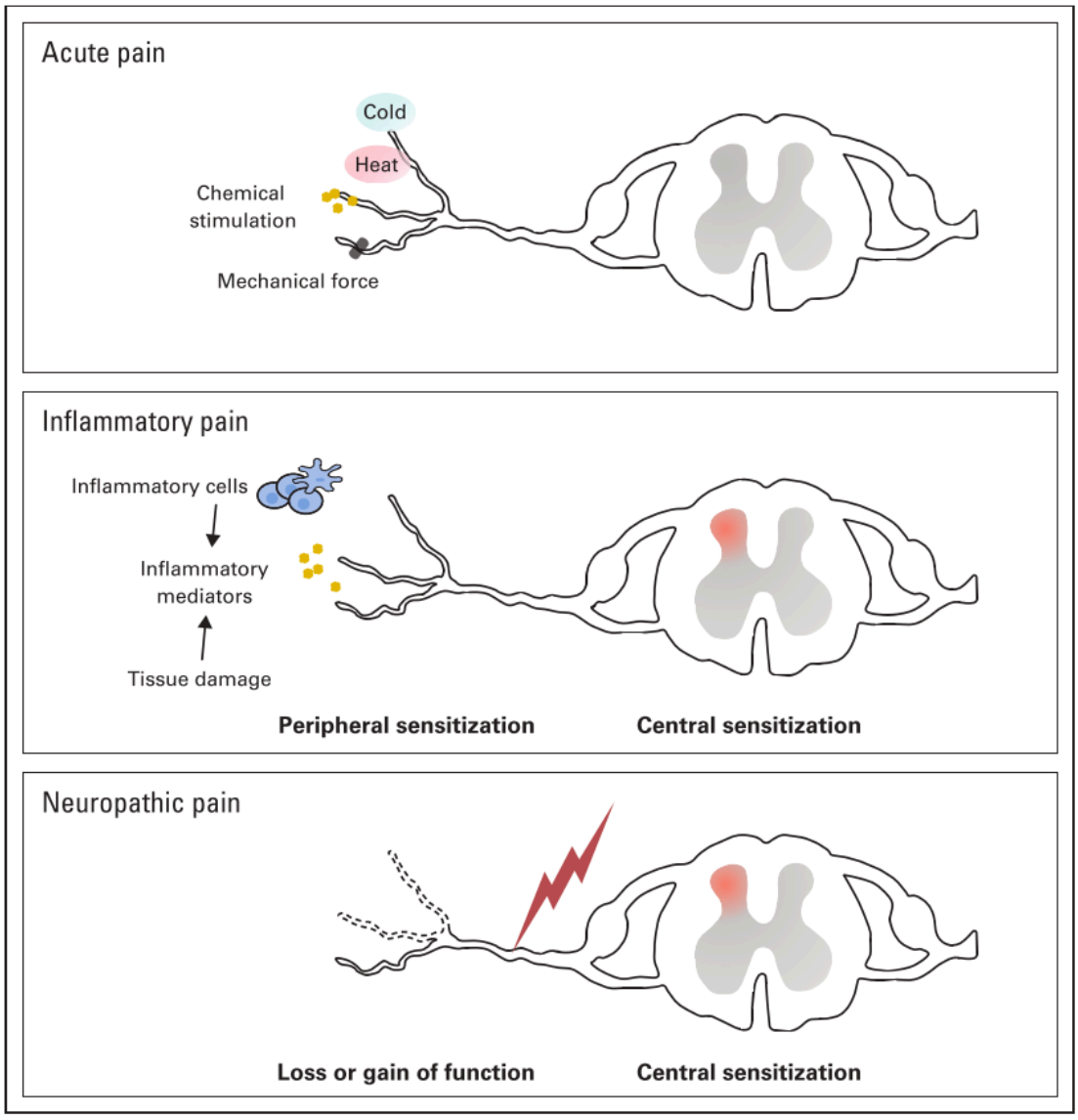


Figure 1.1. Pain mechanisms. Acute pain is a first response to noxious stimuli that can be of chemical, mechanical or thermal origin. This triggers a reflex response and withdrawal from the stimulus but with no lasting damage to the nociceptive system. Inflammatory pain involves inflammatory mediators released in response to tissue damage sensitizing the nociceptive system. Lesions to peripheral or central neurons can cause neuropathic pain. Figure taken from (Falk and Dickenson 2014).

1.1.2 The primary afferent fibres

The nociceptive system begins with the nociceptor: a primary sensory neuron that becomes activated in response to stimuli capable of causing tissue damage (Sherrington 1906). Nociceptors are polymodal sensory neurons that can respond to multiple stimulus modalities of mechanical, thermal and chemical origin. Nociceptors selectively detect and respond to potentially harmful stimuli as they are only activated once the intensity reaches the noxious range due to their biophysical properties (Basbaum et al 2009). The sensory nerve ending of nociceptors are dispersed throughout the body

innervating skin, muscle, joints and internal organs (Julius and Basbaum 2001). The nociceptors have a peripheral axonal branch innervating their target organ in the periphery, while the central axonal branch innervates the spinal cord (Basbaum et al 2009).
The dorsal root ganglia (DRG) are located peripherally alongside the spinal cord, they contain the cell bodies of nociceptors dispersed through the body and directly synapse onto spinal neurons (Lopes et al 2017). The trigeminal ganglion are localized at the base of the skill and contain the cell bodies for nociceptors located in the face, which then project to the brainstem or upper regions of the spinal cord (Lopes et al 2017).

The nociceptors can be grouped into three populations based on their anatomical and functional properties as shown in Table 1.1. The Aδ fibres are a type of thinly myelinated primary afferent fibre found in the periphery. The Aδ afferent fibres can be further subdivided into two main classes. Firstly, the Type I Aδ fibres are also known as the high-threshold mechanical receptors. They respond to mechanical stimuli but have a high threshold for thermal stimulation of >50°C. However, these fibres can become sensitised to both tissue injury and continued thermal stimulation, such that if an intense heat stimulus is maintained the Type I Aδ afferents will begin to respond to lower temperatures (Basbaum et al 2009). The Type II Aδ fibres have a lower thermal threshold but a much higher mechanical threshold. The Aδ fibres are thought to mediate the first response to noxious stimuli, mediating an acute and sharp pain. In summary, type II Aδ fibres are responsible for the first response to acute heat and the type I Aδ fibres trigger a rapid response to intense mechanical stimulation.

The C fibres are a further subtype of peripheral nociceptor, which are unmyelinated, have a small diameter and a slow conduction velocity of <1.4m/s (Harper and Lawson 1985). There are also two major classes of C fibres, which are distinguished by their biochemical and anatomical differences (Snider and McMahon 1998). Firstly is the peptidergic population, they contain the neurotransmitter substance P and express the tyrosine kinase A (TrkA) receptor, which nerve growth factor (NGF) binds to with high affinity (Julius and Basbaum 2001. The second non-peptidergic population can be labeled with the α-D-galactosyl binding lectin IB_4 and express the ATP-gated ion channel $P2X_3$, indicating that these nociceptors use ATP as a neurotransmitter for transmitting the pain signal (Burnstock 2000). C fibres are responsible for a dull and diffuse pain response, which follows the rapid, sharp pain mediated by Aδ fibres.

There is an additional type of peripheral afferent, the Aβ fibre, which has a larger diameter, is highly myelinated and a fast conduction velocity of >14m/s (Harper and Lawson 1985). The Aβ fibres have a low threshold for activation and so respond to light brush and innocuous mechanical stimulation. Under normal conditions the Aβ fibres are not involved in the pain sensation but are believed to be recruited in sensitising pathological conditions (Woolf and Doubell 1994)(Julius and Basbaum 2001).

Furthermore, there are also silent nociceptors which are heat responsive but mechanical insensitive (Basbaum et al 2009). However, they can become sensitive to mechanical stimulation in a state of tissue injury (Julius and Basbaum 2001). These silent afferent fibres are more responsive to chemical stimuli than the polymodal C fibres and so when chemical mediators are released due to an inflammatory state, their properties can become altered and they can be activated by mechanical stimulation too (Schmidt et al 1995).

Table 1.1. Populations of nociceptors based on their conduction velocity, morphology and response characteristics.

Fibre Type	Diameter	Myelination	Conduction velocity (m/s)	Stimuli	Sensation in normal state	Sensation in injured state
Aβ	Large	Myelinated	>14	Light touch and brush	Innocuous	Pain (allodynia)
Aδ Type I	Medium	Partly Myelinated	2.2-8	Noxious mechanical	Nociceptive	Exaggerated pain (hyperalgesia)
Aδ Type II	Medium	Partly Myelinated	2.2-8	Noxious thermal	Nociceptive	Exaggerated pain (hyperalgesia)
C	Small	Unmyelinated	<1.4	Noxious mechanical, thermal and chemical	Nociceptive	Exaggerated pain (hyperalgesia)

1.1.3 Detection of noxious stimuli and transduction of the pain signal

The ability to react and respond to potentially injurious external stimuli is a process crucial to survival. The pain experience begins in the periphery as the nociceptors detect potentially harmful stimuli. The primary afferent fibres found in the periphery are

capable of responding to various stimulus modalities dependent on the types of receptors they possess, which are found on unencaspsulated nerve endings. Receptors in the periphery respond to noxious stimuli to create a generator potential, if the generator potential is of sufficient magnitude it can be amplified to an action potential through the opening of sodium, calcium and potassium channels. As such, activation of receptors results in either an influx or efflux of cations across the plasma membrane and subsequent depolarization or hyperpolarization of the afferent respectively. Once the generator potential is converted to electrophysiological activity, it can be transduced to the spinal cord and higher centres (Bridgestock and Rae 2013). The receptors and channels that mediate membrane depolarization of the nociceptor and the subsequent transduction of the pain signal will be discussed in greater detail in this section.

1.1.3.1 TRP Channels

Heat sensitive ion channels found on primary afferent nerve endings mediate the detection of thermal stimuli (Tominaga and Caterina 2004). Cesare and McNaughton provided the first evidence of this by demonstrating that application of pulses of noxious heat to a subpopulation of nociceptors isolated from the DRG rapidly activated inward currents (Cesare and McNaughton 1996). The authors proposed that the equilibrium between the ion channels closed and open states is dependent on a large entropy difference. They suggested that elevations in temperature must change the heat sensitive ion channels from an ordered (closed) to a more disordered (open) state (Cesare er al 1999). Due to the transient opening and closing states the channels were considered Transient Receptor Potential (TRP) channels.

The TRP channels are vital components of our sensory systems being expressed almost ubiquitously on peripheral nociceptors and responding to temperature, touch, pain, osmolarity and other stimuli (Clapham 2003). The TRP channels are a large family of voltage-gated ion channels; to date 28 mammalian isoforms have been identified that can be subdivided into 7 families (O'Neill et al 2014). Six transmembrane domain (TMD) polypeptide subunits make up the TRP channels, these subunits assemble as tetramers and form pores in the cell membrane (O'Neill et al 2014). Voltage-gated channels function by using a charged gating unit in the membrane field that can be displaced in response to changes in membrane potential, this induces a conformational changed that gates the channel opened or closed (Cesare et al 1999). The opening of the TRP ion channel results in the entry of cations (Na^+ and Ca^{2+}) into the neuron, while the TRP

channels are unselective they have been shown to have a higher affinity for Ca^{2+} (Cesare et al 1999, O'Neill et al 2014).

Humans are capable of sensing a wide range of external temperatures spanning cold to heat, but when temperatures begin to exceed 43°C or drop below 15°C not only a thermal sensation is evoked but also one of pain (Tominaga and Caterina 2004). The thermo-TRP channels are activated over a distinct range of both noxious and innocuous temperatures. The thermo-TRP channels include TRPV1-4, TRPM2, TRPM4, TRPM5. TRPM8 and TRPA1 are included in the thermo-TRP channel category as they are proposed to be responsible for the transduction of cold stimulation in primary afferents (Kobayashi et al 2005a). The thermo-TRP channel with the highest threshold for activation is the TRPV2 channel with an activation threshold of 52°C (Caterina et al 1999).

The activation of the TRPV1 receptor is proposed to lead the perception of heat pain as it is the first receptor to detect noxious heat with an activation threshold of >43°C (Dhaka et al 2006). This suggests that the TRPV1 channel is capable of exciting Aδ and C afferents to send pain signals to the dorsal horn of the spinal cord. TRPV1 can be activated by noxious heat, protons (pH < 5.2), endogenous lipids, inflammatory mediators and capsaicin – the pungent ingredient in hot chilli peppers (O'Neill et al 2014). Upon detection of noxious heat or capsaicin, the opening of the outwardly rectifying, non-selective cation channel TRPV1 evokes membrane currents. However, despite the channel being non-selective, it has been shown to have a high preference for Ca^{2+} (Caterina et al 1997). To confirm the involvement of the TRPV1 channel in thermal nociceptive function, a TRPV1 knockout mouse was generated (Caterina et al 2000). These mice displayed deficient behavioural responses to capsaicin, heat and protons. However, the knockout mice showed normal responses to noxious mechanical stimulation, suggesting that nociceptors require alternative molecular mechanisms to respond to noxious mechanical stimulation.

The speed required for the detection of harmful mechanical stimuli is vital for protection from environmental dangers suggesting that, like our responses to thermal stimuli, ion channels are opened in response to mechanical forces (Brierly et al 2011). The TRPA1 channel has been proposed as the candidate receptor responsible for our response to noxious mechanical stimulation (Vilceanu and Stucky 2010). The TRPA1 receptor is expressed in a subset of small diameter neurons, predominantly

unmyelinated C fibres that co-express the TRPV1 channel. TRPA1 can also be found in larger diameter neurones but its functional expression is more abundant in small fibres (Kerstein et al 2009).

To investigate whether the TRRPA1 channel played any role in mechanical responses, Kwan and collaborators generated a mouse model in which essential exons required for the proper function of the TRPA1 gene were deleted (Kwan et al 2006). The authors demonstrated that TRPA1 knockout animals displayed deficient behavioural responses to punctate mechanical stimuli, cold stimulation (0°C), and mustard oil (Kwan et al 2006). Following this study, to directly determine the contribution of TRPA1 to mechanically evoked responses at the level of the nociceptor, Kerstein et al used an *ex-vivo* skin preparation with pharmacological blockade of the TRPA1 receptor. They found a markedly reduced number of action potentials were fired in C fibres in response to mechanical stimulation when the TRPA1 channel was blocked (Kerstein et al 2009).

The use of whole cell patch clamping has further confirmed the TRPA1 receptors role in mechanical sensation. Neurons cultured from mice with TRPA1 deletion and pharmacological blockage of TRPA1 with an antagonist were shown to reduce mechanically activated intermediate adapting current in small diameter DRG neurons (Brierly et al 2011). Similarly, overexpressing the TRPA1 channel in neurons through transfection of a plasmid increased the current amplitude to mechanical stimulation (Brierly et al 2011). The specific responses to mechanical stimulation of nociceptors, depending on the expression levels of TRPA1, was investigated further by Vilcenau and Stucky when they found that significantly more IB_4 negative neurons (non-peptidergic) responded to mechanical stimulation than IB_4 positive (peptidergic) (Vilcenau and Stucky 2010). They found that slowly adapting currents in response to mechanical stimulation were completely abolished in IB_4 negative neurons in TRPA1 knockout mice, and in wild-type mice in the presence of a TRPA1 antagonist. Interestingly the authors also found that slowly and intermediate adapting currents are non-cation specific while rapidly adapting currents are carried solely by Na^+ ions. This suggests that mechanical responses utilize Ca^{2+} and Na^+ signaling. Taken together, all of the studies discussed above demonstrate the importance of the TRPA1 channel in the transduction of nociceptive information in response to mechanical stimulation.

1.1.3.2 Voltage gated sodium channels

Upon detection of noxious stimuli, the receptors on the free nerve endings of nociceptors become activated leading to membrane depolarization; it is this encoding of action potentials that sends the pain signal. The generation and propagation of action potentials is dependent upon voltage-gated sodium channels. There are four voltage-gated sodium channel subtypes which are particularly interesting to the pain pathway due to their expression on nociceptors, the channels sensitive to the neurotoxin tetrodotoxin (TTX-S), $Na_v1.3$ and $Na_v1.7$ and the tetrodotoxin-resistant (TTX-R) channels $Na_v1.8$ and $Na_v1.9$ (Wood and Momin 2008).

The $Na_v1.3$ channel subtype is highly expressed during development but there is little expression in the adult sensory neurons, however it can be upregulated along the pain pathways in response to injury (Waxman et al 1994). Waxman and collaborates demonstrated that mRNA levels of $Na_v1.3$ were highly expressed in sensory neurons following both axotomy and inflammation. The $Na_v1.3$ channel has a rapid recovery from inactivation so this re-expression could suggest a role for this channel in high frequency firing and bursts of action potentials in chronic pain states (Wood and Momin 2008).

The $Na_v1.7$ channel produces a fast activating and inactivating, slow-repriming current suggesting this channel is responsible for amplifying the small subthreshold depolarizations known as generator potentials (Dib-Hajj et al 2010). This proposes that this channel acts as the threshold channel for firing action potentials and regulates the excitability of nociceptors (Dib-Hajj et al 2013). A mutation in the SC9NA gene which encodes the $Na_v1.7$ channel. results in a phenotype of a complete inability to perceive any form of pain (Cox et al 2006). Therefore, the $Na_v1.7$ channel appears to be an essential requirement for nociception.

The pain signal then recruits the $Na_v1.8$ channel that is present along peripheral axons and free nerve terminals in the skin. The $Na_v1.8$ channel contributes most of the sodium current underlying the action potential and accounts for most of the current in latter action potential spikes (Dib-Hajj et al 2010).

Finally, the $Na_v1.9$ channel is expressed in small diameter, non-peptidergic DRG neurons. In humans, the $Na_v1.9$ channel activates at approximately -80mV, very close to

the resting membrane potential and its very slow inactivation leads to a persistent current. This $Na_v1.9$ channel is not thought to contribute to the action potential upstroke but to enhance and prolong the response to subthreshold depolarizations (Dib-Hajj et al 2010).

1.1.3.3 Voltage gated calcium channels

Similarly to sodium ions, the rapid entry of calcium into a cell causes changes in membrane potential, and contributes to repetitive firing and electrical excitability of nociceptors (Snutch 2006). Voltage gated calcium channels (VGCC) can open following membrane depolarization and are responsible for the entry of calcium into the neuron along its electrochemical gradient (Simms and Zamponi 2014). Following the activation of nociceptors and the resulting action potential mediated by sodium channels, the electrical signal is also translated into a chemical one mediated by neurotransmitters. Calcium entry into presynaptic nerve terminals is the initial trigger for the release of neurotransmitters into the synapse; this depends on calcium dependent fusion of synaptic vesicles (Snutch 2005). The N-type voltage gated calcium channels (VGCC) are highly expressed in C and Aδ primary afferents, with the Cav2.2 channel being the most abundant VGCC in nociceptors (Woolf and Ma 2007). It has been demonstrated that high voltage activated calcium currents contribute much less than sodium to the inward charge during the upstroke of the nociceptor action potential (Blair and Bean 2002). Indeed, high threshold calcium channels typically activate at membrane potentials of approximately -30mV. However, the action potentials had a prominent shoulder during the falling phase, which is characteristic of nociceptors, and they found the high voltage activated calcium currents contributed significantly to this part of the action potential (Blair and Bean 2002).

The electrical activity of a neuron is dependent upon the state of voltage-gated channels on their membrane. The opening of voltage-gated sodium and calcium channels and the subsequent entry of cations into the neuron propagate an action potential. This can trigger membrane depolarization and the opening of voltage-gated continues along the length of the neuron allowing the pain signal to be transduced as electrical activity.

1.1.4 Peripheral sensitization

The properties of primary afferent nociceptors can be altered upon injury or inflammation, this can result in increased spontaneous firing, changes in their

conduction properties, and increased neurotransmitter release. The increased excitability of primary afferents is thought to contribute to persistent and chronic pain states and is known as peripheral sensitization.

1.1.4.1 Inflammatory mediators sensitising primary afferents

Peripheral sensitisation occurs most often when tissue damage causes inflammatory associated changes in the environment of the nociceptor. In addition to the TRP, voltage gated sodium and calcium channels mentioned above, the primary afferents express a myriad of other receptors. This includes voltage-gated and voltage-insensitive potassium (K^+) channels, tyrosine kinase receptors activated by growth factors, G-protein coupled receptors (GPCRs), and ASIC channels activated by protons. The inflammatory mediators released due to tissue injury can activate and sensitise the array of receptors found on primary afferent nerve endings.

Upon tissue injury, there is a surge of inflammatory mediators released by blood vessels, resident mast cells, recruited immune cells, epithelial cells, Schwann cells and fibroblasts surrounding the nociceptor (Basbaum et al 2009). The sensitising substances released by these sources in response to inflammation are often referred to as the 'inflammatory soup'. This 'inflammatory soup' contains a wide array of components including neurotransmitters, peptides such as substance P, calcitonin gene-related peptide (CGRP) and bradykinin, prostaglandins, neutrophins, cytokines and chemokines (Basbaum et al 2009)(Gold and Gebhart 2010). The multiple components of the 'inflammatory soup' released due to tissue injury and the myriad of receptors they can potentially sensitise are shown in Figure 1.2. Upon release, these inflammatory agents can either directly activate receptors to modulate their responses, or function by binding to their own receptors on primary afferents which subsequently alters other receptor properties via activation of downstream intracellular signaling pathways (Basbaum et al 2009).

Preventing the sensitization of primary afferents caused by this 'inflammatory soup' became an attractive target for analgesics. A good example of this is the

non-steroidal anti-inflammatory drugs (NSAIDs), which function by preventing the synthesis of these inflammatory mediators. An example of this is ibuprofen and aspirin that work by inhibiting cyclooxygenases (Cox-1 and Cox-2), which are required for the synthesis of prostaglandins (Basbaum et al 2009).

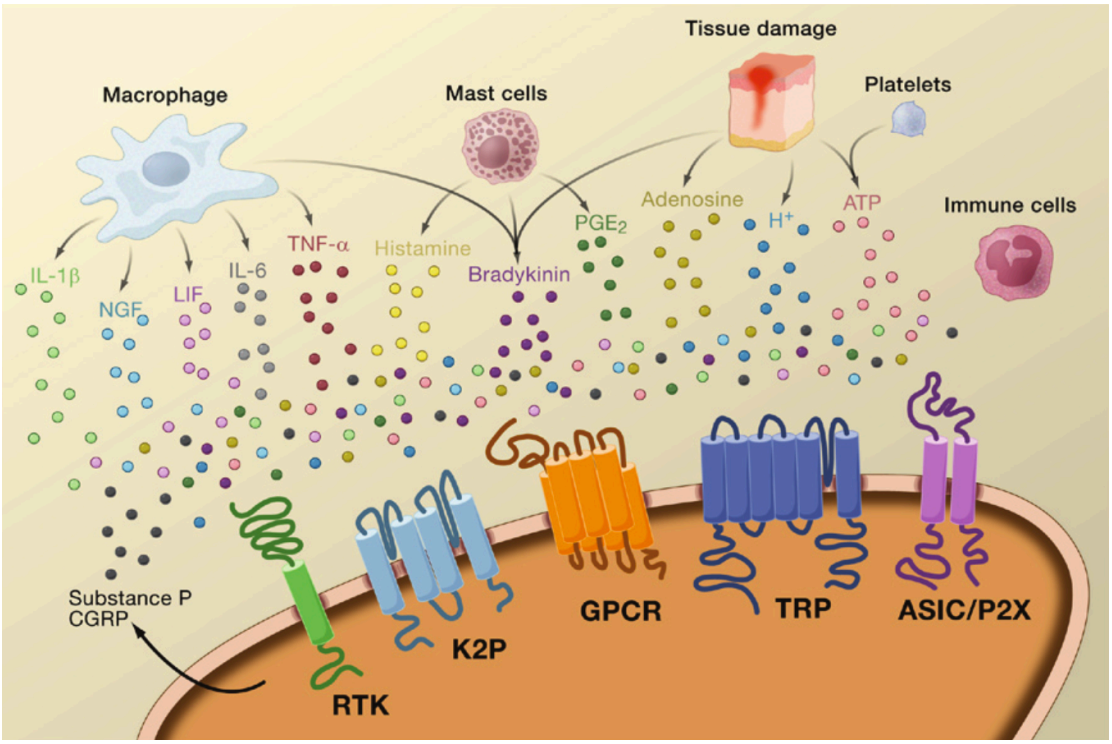


Figure 1.2. The release of inflammatory mediators in response to tissue injury and the potential receptors they sensitize. Upon tissue injury, both neuronal and non-neuronal cell types that either reside within or infiltrate the nociceptor environment release a wide array of inflammatory components. These sensitising components can activate the myriad of receptors expressed on primary afferent nerve endings. (IL-1β –Interleukin 1β, NGF – nerve growth factor, LIF – leukemia inhibitory factor, IL-6 – Interleukin-6, TNF-α – tumour necrosis factor α, PGE_2 – Prostaglandin E2, ATP – Adenosine triphosphate, CGRP – calcitonin gene related peptide, RTK – tyrosine kinase receptor, K2P – Two pore potassium channel, GPCR – G-protein coupled receptor, TRP – Transient receptor potential channel, ASIC – acid-sensing ion channel, P2X – ATP gated ion channel. Figure taken from (Basbaum et al 2009).

Firstly, components of the inflammatory soup can directly modulate receptor responses by functioning as direct positive allosteric modulators of ion channels. Cytokines are one of the components released due to tissue damage, the main ones being interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumour necrosis factor α (TNFα) (Zhang and An 2007). Whilst these cytokines have a direct action on nociceptors they are also responsible for further potentiating the inflammatory response by causing an increased production of further inflammatory mediators (Zhang and An 2007). As many channels found in the primary afferent nerve ending are voltage dependent, the resting membrane potential is a very important factor influencing transduction of the pain signal. Under normal

conditions there is little spontaneous activity in nociceptors due to a large K^+ conductance, which works to oppose the high membrane permeability to depolarizing Na^+ and Ca^{2+} (Gold and Gebhart 2010). Therefore, a direct modulation of inflammatory mediators on potassium channels may influence the resting membrane potential and alter the level of stimulation required for spike initiation.

Metabotropic receptors such as GPCRs signal through second messenger pathways, therefore activation of these receptors initiates a cascade of intracellular signaling events, and the intracellular substances that become activated can subsequently modulate the activity of other receptors within the plasma membrane. Prostaglandin E2 (PGE2) binds to GCRPs to increase the levels of cyclic AMP (cAMP) within nociceptors to subsequently activate protein kinases . Protein kinase A (PKA) has been shown to phosphorylate TTX-R Na^+ channels which shifts their voltage dependence in the hyperpolarizing direction, reducing the stimulation needed for initiation of an action potential (Julius and Basbaum 2001). Bradykinin can activate GPCRs and results in activation of phospholipase C (PLC), this intracellular increase in PLC can modulate TRPA1 receptors and evoke mechanical hypersensitivity (Kwan et al 2006).

One receptor particularly susceptible to modulation via second messenger intracellular cascades is the TRPV1 channel as shown in Figure 1.3. The TRPV1 channel is associated with a number of intracellular proteins; one example is phosphatidyl inositol 4,5 biphosphate (PIP_2), which is attached to TRPV1 through its C terminal domain and inhibits channel gating (Prescott and Julius 2003). The activation of intracellular PLC through GPCRs can cause cleavage of PIP_2 from the plasma membrane and activates the TRPV1 receptor (O'Neill et al 2014). A further downstream consequence of the activation of intracellular PLC is an increase in intracellular calcium through a diglyceride (DAG) and Inositol 1,4,5-triphosphate (IP_3) mediated pathway, causing further activation of protein kinases (Basbaum et al 2009, Gold and Gebhart 2010). The activation of GPCRs can also cause activation of adenylate cyclases and subsequent activation of PKA and PKCε through a cAMP-activated guanine exchange factor (EPAC) dependent pathway (Gold and Gebhart 2010). In addition, activation of Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) can be caused by an increase in intracellular Ca^{2+}. TRPV1 can become sensitized by phosphorylation via both of these protein kinases (Gold and Gebhart 2010). Finally, activated second messenger cascades can cause TRPV1 to be translocated to the plasma membrane, further increasing nociceptor excitability.

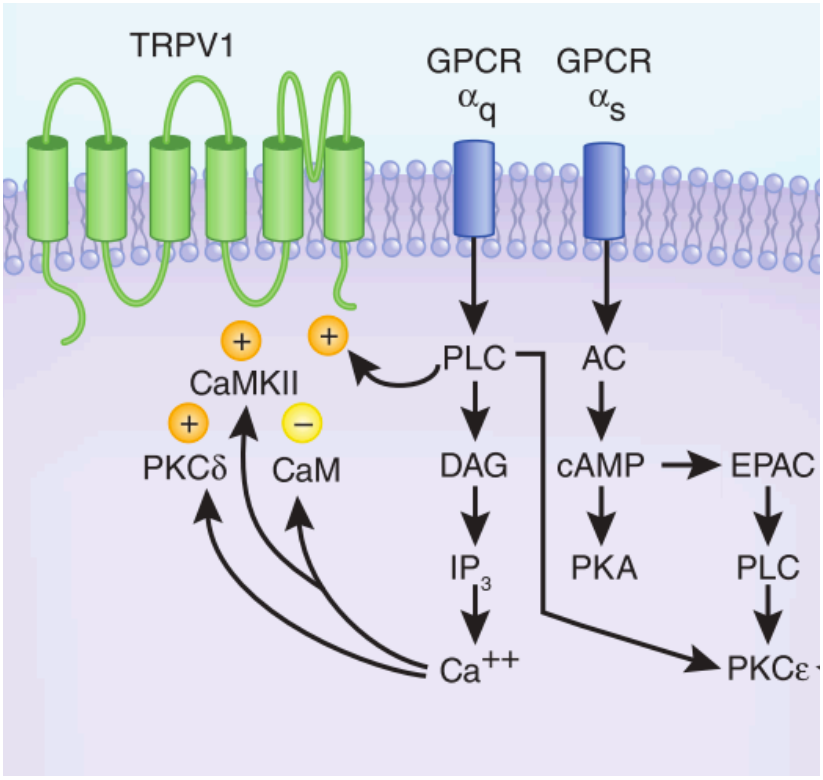


Figure 1.3. Sensitisation the TRPV1 channel due to the activation of GPCRs and the subsequent activation of intracellular second messenger cascades. The activation of GPCRs by inflammatory mediators activates intracellular second messenger cascades resulting in activation of phospholipase C (PLC), protein kinases and an increase in intracellular Ca^{2+}. All of these intracellular factors can sensitise the TRPV1 channel. CaMKII - Ca^{2+}-calmodulin-dependent protein kinase II, PKC – protein kinase C, CaM – Calmodulin, PLC – phospholipase C, DAG – diglyceride, IP_3 – Inositol 1,4,5-triphosphate, AC – adenylate cyclase, cAMP – cyclic AMP, PKA – protein kinase A, EPAC – cAMP activated guanine exchange factor, PKCε – protein kinase C ε. Figure adapted from (Gold and Gebhart 2010).

As there are multiple sources of inflammatory mediators, a vast array of receptors they can act upon, and a positive feedback response to activation of receptors on primary afferents, it is unsurprising that tissue injury has a rapid and intense impact upon nociceptor sensitization and the resultant pain signal. Overall, the increased excitability of primary afferents fibres results in an increased sensitivity and response to mechanical and thermal stimuli.

1.1.4.2 Primary hyperalgesia and allodynia

Tissue damage and the subsequent inflammatory response cause excessive activation of nociceptors and an increased sensitivity to pain. There are two main clinical symptoms of chronic or persistent pain, which are allodynia and hyperalgesia. Firstly, allodynia is characterized by a painful response to innuocuous stimuli, specifically a stimulus that would not usually be encoded by nociceptors. Therefore, the Aβ primary afferents that usually encode innocuous light touch and brush are thought to be responsible for

allodynia (Woolf and Doubell 1994). In contrast, hyperalgesia is an increased response to noxious stimuli (Jensen and Finnerup 2014). In persistent pain states, the curve that describes the relationship between stimulus strength and the pain response is shifted leftwards, as shown in Figure 1.4, as patients become more sensitive to mechanical and thermal stimuli (Jensen and Finnerup 2014). The consequence of this leftward shift is that patients show allodynia and hyperalgesia as shown in Figure 1.4. Mechanical and thermal hyperalgesia can be demonstrated in a rat model of acute inflammation induced in the rat paw with an injection of complete Freunds adjuvant (CFA) (Andrew and Greenspan 1999). Single fibre recordings taken from the sciatic nerve in these animals showed a sensitised response to suprathreshold mechanical and thermal stimuli for both A and C fibres, but mechanical sensitization was greater in A fibres. The authors concluded that the primary hyperalgesia caused by peripheral inflammation was due to the sensitised state of the nociceptors (Andrew and Greenspan 1999).

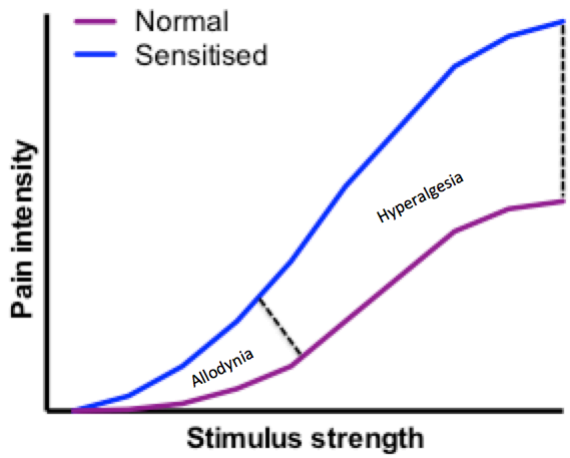


Figure 1.4. Stimulus strength and the resulting pain response in both the normal and sensitised state. The Purple curve shows the stimulus-pain relationship expected in a normal subject. The blue curve shows the left-ward shift you would expect to the stimulus-pain relationship in a patient sensitised to pain. The bottom of the curves shows allodynia, where low strength stimulation that would usually be innocuous causes a larger pain response. The difference in the tops of the curves indicates hyperalgesia, where noxious stimulation causes a greater pain intensity.

1.1.4.3 Nerve Growth Factor

Of particular interest in inflammatory conditions such as osteoarthritis, is the contribution of the neurotrophin NGF to peripheral sensitization. NGF is a vital component of the 'inflammatory soup' produced in response to tissue damage and can act as a sensitizing agent at peptidergic C fibres (McMahon 1996). NGF can bind to two receptors; the TrkA receptor selectively binds NGF with high affinity while the p75

receptor will bind any neutrophin but has a low affinity for NGF (Chao 2003). Upon the binding of growth factors to TrkA, receptor dimerization is induced and the receptor becomes activated (Lemmon and Schlessinger 2010).

NGF has been shown to contribute to hypersensitivity of nociceptors especially through sensitization of TRPV1. The TrkA receptor is frequently co-expressed on nociceptors with the TRPV1 channel. It has been shown that NGF rapidly potentiates the responses of nociceptors to capsaicin; specifically NGF enhanced the rise of intracellular Ca^{2+} observed in primary afferents following application of capsaicin (Bonnington and McNaughton 2003). Furthermore, the authors found a specific inhibitor of phosphoinositide (PI3K) totally abolished this effect. The authors suggest that through binding TrkA, NGF causes activation of intracellular PI3K, which activates CaMKII, this subsequently activates PKC and hence can phosphorylate the TRPV1 receptor (Bonnington and McNaughton 2003). They propose this pathway is a crucial initial step in sensitization of TRPV1 in response to NGF. In addition to this second messenger cascade, NGF binding TrkA receptors can stimulate intracellular PLC signaling (Chuang et al 2001). Further co-immunoprecipitation studies showed that TRPV1 forms a complex in the plasma membrane with TrkA and PLC. Once activated, PLC can mediate the hydrolysis of PIP_2 from TRPV1 to sensitize the receptor (Chao et al 2003). Overall, the binding of NGF to TrkA receptors on the nociceptor membrane can exacerbate peripheral sensitisation.

Additionally, increased levels of NGF induce nociceptor sensitization as it is retrogradely transported to the nucleus where it can influence gene expression. Specifically, NGF promotes and increases expression of the pronociceptive proteins substance P and the $Na_v1.8$ channel. This change in gene expression and subsequent increase in $Na_v1.8$ levels can lower the level of stimulation needed for an action potential to fire and hence enhances the excitability of the nociceptor (Basbaum et al 2009).

Increased levels of NGF at the site of inflammation is linked to an increased level of pain, making NGF particularly interesting with regard to osteoarthritis, an inflammatory condition of the joints. Several NGF neutralizing antibodies have been produced and are being tested for their effectiveness at reducing peripheral sensitisation. Tanezumab is a humanized IgG2 monoclonal antibody that functions by blocking the interaction of NGF with both the TrkA and p75 receptor. In clinical trials Tanezumab reduced joint pain in patients with moderate to severe osteoarthritis of the knee joint (Lane et al 2010).

1.1.5 The dorsal horn

The dorsal horn contains the first synapse of ascending pathways that subsequently transmit the pain signal to higher centres, making it the first relay site where nociceptive information converges and is transmitted from the periphery to the brain (D'Mello and Dickenson 2008). The cell bodies in the DRG split into two main branches, the peripheral branch innervates external tissue such as skin and detects noxious stimuli, while the central branch of axons travels through a dorsal root to enter the dorsal horn of the spinal cord. Here the peripheral afferents form synapses with second order neurons as shown in Figure 1.5 (Bridgestock and Rae 2013). Within the spinal cord, the pain signal can be modulated by a vast array of neuron types, such as interneurons or descending projections, found throughout the dorsal horn. Additionally, the primary afferents can form synapses with second order neurons that transmit the nociceptive information to the ventral horn to mediate a nocifensive reflex (Todd 2010).

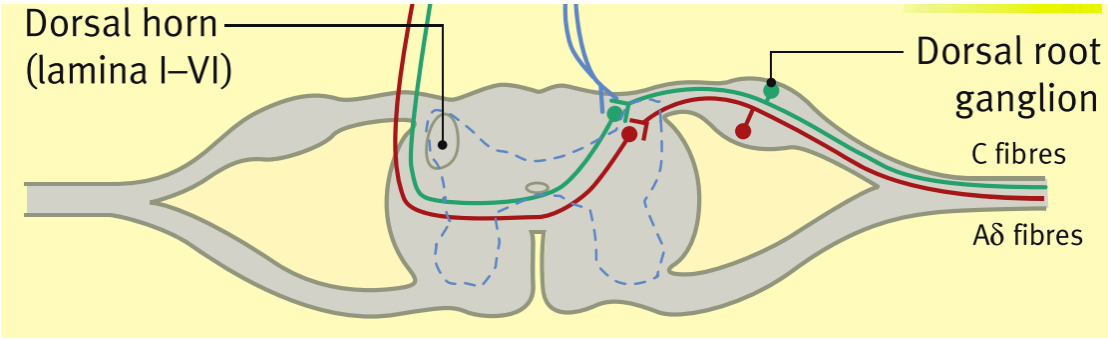


Figure 1.5. The dorsal horn. Primary afferent nociceptors converge onto the dorsal horn of the spinal cord via dorsal root ganglia. Upon termination in the spinal cord, the primary afferent fibres form synapses with second order neurons that transmit the signal to higher centers in the brain. Figure taken from (Bridgestock and Rae 2013).

The dorsal horn is divided into ten lamina starting with the most superficial layer, lamina I, and moving ventrally through to lamina X (Figure 1.7). A brief summary of the lamina arrangements is shown in Table 1.2. The functional class and sensory modality of the primary afferents determines where they terminate within the dorsal horn. The lumbar region of the spinal cord in which the peripheral nociceptors terminate depends on the region of the body they innervate. Aβ fibres enter the dorsal horn through the dorsal root, where the main axon can branch into collateral fibres which then terminate throughout lamina II to lamina V (Todd 2010). Aδ primary afferents terminate mainly in the superficial lamina I and II but some axonal branches pass ventrally to terminate in lamina IV and V of the deep dorsal horn (Light and Perl 1979). Mainly, C fibres terminate in the superficial dorsal horn in lamina I and II (Todd 2010).

The exact sites at which peripheral afferent fibres terminate within the dorsal horn depend upon their biochemical properties. Specifically, the non-peptidergic C fibres project to the dorsal part of lamina IIi. Meanwhile the peptidergic fibres arborize mostly in lamina I and IIo, however they have some further terminations in the deeper dorsal horn, starting at lamina IIi and scattered through to lamina V. The main termination sites of the primary afferents are shown in Figure 1.6.

The primary afferents form synapses with two major types of second-order neurons in the dorsal horn, these neurons are the nociceptive specific (NS) neurons and the wide dynamic range (WDR) neurons, which are responsible for transmitting the pain signal to higher brain centres (Figure 1.6). The NS neurons specifically detect noxious stimuli and are mainly located in lamina I (D'Mello and Dickenson 2008). The WDR neurons can detect a range of stimulations, from innocuous stimulation such as light touch or brush to stimulations in the noxious range. The WDR neurons are located in lamina V and receive convergent input from Aβ, Aδ and C fibres (D'Mello and Dickenson 2008).

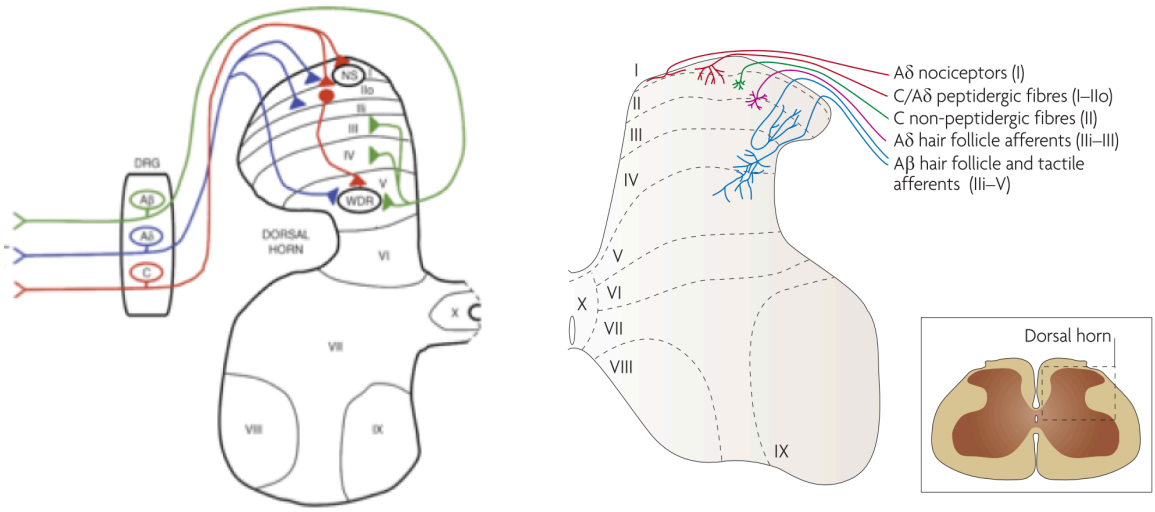


Figure 1.6. The location of nociceptive specific and wide dynamic range neurons and the termination zones of primary afferents in the dorsal horn of the spinal cord. The Aβ fibres have termination zones extending from lamina II to lamina V. The Aδ primary afferents terminated mainly in the superficial lamina I and II. The peptidergic and non-peptidergic C fibres have distinct termination patterns with the peptidergic fibres terminating more superficially in the dorsal horn than the non-peptidergic C fibres. Figure taken from (Todd 2010 and D'Mello and Dickenson 2008).

Another major component of the dorsal horn are the interneurons, which have axons that remain in the spinal cord and arborize locally to mediate the actions of surrounding neurons. These are particularly abundant in the superficial lamina with interneurons making up almost all of the neurons in lamina II and most of the neurons in lamina I and

III (Todd 2010). The interneurons that are involved in local circuits have axons that terminate in the same segment close to their cell body. Some interneurons also contain axons that extend into other lamina, some of the lamina II interneurons have axons terminating in lamina I or passing ventrally through to lamina III-IV (Todd 2010).

The interneurons can be characterized into excitatory or inhibitory interneurons. The excitatory interneurons use glutamate as their neurotransmitter, whereas inhibitory interneurons use the neurotransmitters GABA and glycine (Todd and Spike 1993). It has been shown using immunocytochemistry that GABAergic inhibitory interneurons are dispersed in large numbers throughout the dorsal horn, whilst the termination sites of gylcinergic interneurons are more common in the deep lamina II-IV. Furthermore, immunostaining studies have shown that GABA and glycine can be co-released from these neurons (Todd and Sullivan 1990, Todd and Spike 1993). Through the release of these neurotransmitters, the interneurons can target other nearby neurons within the dorsal horn to modulate their activity.

Similarly to interneurons, projection neurons can also be found in the dorsal horn concentrated in large numbers in lamina I, with a few scattered through lamina III-VI (Todd 2010). With the use of tract tracing and immunocytochemical techniques, it was demonstrated that lamina I and III projection neurons are densely innervated by pepetidergic primary afferent C fibres containing substance P, indicating they receive nociceptive signals from nociceptors (Todd et al 2002). Neurokinin-1 (NK1) receptors are found on 80% of lamina I projection neurons. The release of substance P into the dorsal horn following noxious stimulation can then activate and internalize NK-1 receptors located on the projection neurons (Mantyh et al 1997, Nichols et al 1999, Suzuki et al 2002) The projection neurons have axons that project to the brain, making them responsible for the transmission of the pain signal from the dorsal horn to the brain. Many of these axons cross the midline and transmit the pain signal rostrally through the contralateral white matter where they subsequently terminate in various brain regions. The brain regions in which the lamina I projection neurons terminate include the caudal ventrolateral medulla (CVLM), the nucleus of the solitary tract (NTS), the lateral parabrachial area (LPb), the periaqueductal grey (PAG) and nuclei in the thalamus (Todd 2010). Therefore the projection neurons represent the relay point responsible for the transmission of the nociceptive signal from peripheral afferent neurons to brain neurons where the information is processed.

Table 1.2. The distribution of neurons in the dorsal horn.

Lamina	Neuronal distribution
I	Contains the highest density of projection neurons in the dorsal horn. However, projection neurons make up only 5% of the total neurons in lamina I. The remaining neurons are interneurons.
II	Contains virtually all unmyelinated interneurons.
III	Mostly contains small myelinated interneurons but also includes a scattering of large projection neurons.
IV-VI	Contains heterogenous neurons of various sizes some of which are projection neurons.

1.1.5.1 Transmission of the pain signal

The depolarization of peripheral sensory fibres leads to activation of VGCCs, specifically the Cav2.2 channel. The subsequent influx of Ca^{2+} initiates the release of neurotransmitters from presynaptic terminals into the spinal cord. All primary afferent fibres release glutamate as the main excitatory neurotransmitter but the peptidergic C fibres also release substance P, CGRP, somatostatin, brain-derived neurotrophic factor (BDNF) and galanin (Dickenson 1995). There are three ionotropic glutamate receptors present in the dorsal horn: the α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid (AMPA) receptor, the N-methyl D-aspartate (NMDA) receptor, and the kainite receptor. The AMPA receptor mediates fast glutamatergic neurotransmission; hence they are responsible for the acute response to the nociceptive signal (Hartmann et al 2004, Keinanen et al 1990). The NMDA receptor has a high Ca^{2+} permeability and is responsible for mediating the slower component of excitatory post-synaptic potentials (Monyer et al 1992)(Bleakman et al 2006). The activation of these post-synaptic receptors on second order neurons in the dorsal horn evokes an influx of Ca^{2+} and allows the transmission of the nociceptive signal to multiple brain regions.

1.1.5.2 The NMDA receptor and wind-up

The lamina V WDR neurons display a unique characteristic known as wind-up. Wind-up occurs when there is rapid and repeated noxious stimulation acting on C fibres, which results in an enhanced output of spinal cord neurons (Dickenson and Sullivan 1987). The NMDA receptor has voltage-dependent sensitivity to magnesium, under normal conditions this channel is closed due to a Mg^{2+} plug. However upon peptidergic C fibres co-releasing neuropeptides, such as substance P and CGRP, along with glutamate, a slow membrane depolarization is maintained and results in removal of the Mg^{2+} plug (D'Mello and Dickenson 2008). Upon removal of the Mg^{2+} block the NMDA channel is opened and

in this configuration is highly permeable to Ca^{2+}, meaning Ca^{2+} floods into the neuron where it can activate effectors and subsequently alter downstream signaling. This causes an increased excitability of the neuron whereby more action potentials are fired in response to subsequent noxious stimulation. Wind-up reflects a form of activity dependent plasticity occurring in the spinal cord and the NMDA receptor has been shown to be sensitised and upregulated in both inflammatory and neuropathic pain states (Bleakman et al 2006, Brown and Krupp 2006).

1.1.6 Central Sensitisation

The sensory processing of noxious stimuli can be amplified in chronic pain states through peripheral sensitisation, as discussed in section 1.1.4, and also through neuronal plasticity in the spinal cord. Neuronal plasticity refers to the ability of neurons to adjust their activities and connections in response to increased stimulation. Ongoing stimulation of the primary afferent C and Aδ fibres can result in the sensitisation of dorsal horn neurons. Neurons are capable of changing their function, chemical profile and structure in response to an intense and continuous barrage of noxious stimulation. This plasticity results in an increase in the efficacy and strength of synapses they form within the dorsal horn. Wind-up demonstrates a form of plasticity in the spinal cord, but it is a short-lived phenomenon that disappears upon removal of the stimulation, when the normal resting membrane potential is restored (Herrero et al 2000). Conversely central sensitisation causes profound changes in sensory processing, which outlasts the period of peripheral stimulation (Woolf 2011, Woolf and Salter 2000).

There are different forms of plasticity within the dorsal horn that can contribute to the overall manifestation of central sensitisation. When the neuronal changes are limited to the activated synapse they are referred to as homosynaptic, when the modulation of responses spreads to adjacent synapses it is heterosynpatic (Woolf and Salter 2000). Long term potentiation (LTP) is a homosynaptic potentiation which occurs due to brief high frequency peripheral inputs. The excitability of synapses can be modulated by pre- and post-synaptic transcriptional changes, this process takes the longest to manifest and can last for days (McMahon et al 2013, Section 1 Chapter 5). Finally there can be a depression of inhibition, mediated by both inhibitory interneurons and descending controls, in the dorsal horn (Rahman et al 2008, Todd 2010).

Similarly to wind-up, activity dependent central sensitization begins with intense stimulation of small unmyelinated nociceptors. The peptidergic C fibres synthesize substance P, BDNF and CGRP, which can be released upon nociceptor activation and ultimately result in the release of the Mg^{2+} plug from post-synaptic NMDA receptors. Activation of the NMDA channel results in a dramatic enhancement in the influx of Ca^{2+} into the neuron (Dickenson and Sullivan 1987). In addition, substance P, BDNF and GCRP are released from pre-synaptic terminals, and can bind to post-synaptic NK-1, Tyrosine kinase B (TrkB) and CGRP receptors respectively (Latremoliere and Woolf 2009). The binding of these neuropeptides to their post-synaptic receptors and an increase in intracellular Ca^{2+} above a sufficient threshold can set off a cascade of intracellular signaling pathways, and this leads to subsequent modifications of other post-synaptic receptors. Activation of intracellular signaling cascades subsequently activates the downstream protein kinases PKA, PKC, CamKII and the nonreceptor tyrosine kinase Src, which can all go on to phosphorylate tyrosine and serine residues on other intracellular proteins (Ji and Woolf 2001). PKA and PKC phosphorylate several serine residues on the C terminus of AMPA and NMDA receptors, this post-translational modification changes channel activity and increases trafficking of these receptors to the membrane. PKC mediated phosphorylation of AMPA receptors and their subsequent insertion into the plasma membrane activates 'silent' glutamatergic synpases between primary afferents and second order dorsal horn neurons (Li et al 1999). Secondly, CaMKII can phosphorylate the Ser831 residue on GluR1 subunits of AMPA receptors, which causes the channel to open in a high conductance state (Latremoliere and Woolf 2009). Furthermore, the conductance properties of NMDA receptors can be modified by phosphorylation of tyrosine residues, mediated by Src (Yu et al 1997, Woolf and Salter 2000). Most of these activated signaling pathways converge to activate the intracellular protein ERK. ERK phosphorylates the Ser616 residue on the voltage-gated potassium channel Kv4.2, which decreases K^+ currents and subsequently increases membrane excitability (Latremoliere and Woolf 2009). Overall, the phosphorylation of membrane receptors and intracellular proteins play a major role in the increased neuronal activity observed in central sensitisation.

The activation of intracellular signaling cascades due to activity dependent central sensitisation can also mediate transcriptional changes. Another function of activated ERK in central sensitisation involves its entry into the nucleus along with other activated transcription factors to drive the expression of genes through binding to their promoter regions. Specifically, when ERK enters the nucleus it phosphorylates the

Ser133 residue on the cAMP response element-binding protein (CREB) (Latremoliere and Woolf 2009). An increased expression of the early response genes c-Fos and Cox-2, and the late response genes NK1 and TrkB have been shown in dorsal horn neurons in response to noxious stimulation and inflammation (Woolf and Salter 2000, Hunt et al 1987). The intracellular mechanisms mediating activity dependent central sensitisation are shown in Figure 1.7.

Long term potentiation (LTP) involves long-term enhancement and structural changes at the level of the synapse (Soderling and Derkach 2000). This phenomenon has been mostly studied in CA1 hippocampal synapses where it has been shown that enhancement of NMDA receptor function mediated by Src phosphorylating tyrosine residues raises intracellular Ca^{2+} and subsequently activates CaMKII and PKC (Bliss and Collingridge 1993). These kinases can increase trafficking of AMPA receptors to the cell surface and enhance the conductance of this channel (McMahon et al 2013, Section 1 Chapter 5). The expression of CaMKII is relatively low in the dorsal horn, therefore the serine/threonine kinase PKC is believed to play the principle role in increasing the excitability of the AMPA receptor for LTP in the spinal cord (Woolf and Salter 2000, Soderling and Derkach 2000).

The final mechanism believed to play a role in central sensitisation is disinhibition, where spinal inhibitory interneurons are suppressed allowing excitatory transmission to be sustained (Todd 2010). This phenomenon has been confirmed using nerve injury models in the rat. Firstly, GABAergic density has been demonstrated to be significantly reduced in lamina I-III of the dorsal horn following injury of the sciatic nerve (Ibuki et al 1997). Yet, some GABAergic immunoreactivity returned several weeks after injury, suggesting there was a downregulation of this neurotransmitter rather than a loss of immunoreactivity due to neuronal death (Ibuki et al 1997). Furthermore, GABA receptor mediated inhibitory post-synaptic currents were found to be decreased in two models of peripheral nerve injury, both chronic constriction and spared nerve injury (Moore et al 2002). However, peripheral nerve injury does cause cell death in the dorsal horn as an increase in the number of apoptotic cells has been shown in the ipsilateral dorsal horn following chronic constriction injury (Whiteside and Munglani 2001). Taken together, the cell death of inhibitory interneurons, or a reduction in GABAergic inhibitory currents could be contributing to the maintenance of central sensitisation (Woolf 2000).

The main symptoms of central sensitisation are secondary hyperalgesia and allodynia. Secondary hyperalgesia occurs due to the increased synaptic efficacy in the dorsal horn, which means that there is an increase in the number of action potentials fired in response to noxious stimulation, and subsequently a heightened response. While this increased response is found in central terminal synapses for nociceptors that are activated by the initiating stimulus, low threshold Aβ fibres that would not respond in a normal setting also begin to activate second order dorsal horn neurons (McMahon et al 2013, Section 1 Chapter 5). As such, the NS neurons found in superficial lamina begin to function as WDR neurons responding to both noxious and innocuous simulation (Latremoliere and Woolf 2009). In response to injury, the central axons of myelinated Aβ fibres can sprout from their normal termination site in the deeper lamina of the dorsal horn into the superficial lamina and enter termination sites normally restricted to C and Aδ fibres (Baba et al 1999). The NS neurons now have a reduced activation threshold; meaning innocuous stimuli in the periphery can now elicit a noxious response, this is known as secondary allodynia. Furthermore, Aβ fibres have been shown to start expressing and releasing substance P following nerve injury (Malcangio et al 2000). This allows Aβ fibres to contribute to central sensitisation further by increasing the activation of sensitising intracellular signaling cascades (Malcangio 2000). In an inflammatory arthritis model displaying secondary hyperalgesia, stimulation of A nociceptors results in c-Fos like immunoreactivity in lamina I and IV-V of the dorsal horn, suggesting sensitisation of dorsal horn neurons in these lamina contribute to secondary sensitivity (Hsieh et al 2015). Additionallt, the ablation of NK1 receptor-expressing projection cells in the superficial lamina of the dorsal horn with a substance P saporin conjugate prevents the development of hyperalgesia in models of inflammatory and neuropathic pain (Mantyh et al 1997, Nichols et al 1999). Overall, an expansion of the receptive fields of dorsal horn neurons occurs due to the recruitment of subthreshold synaptic input meaning that areas adjacent to the site of injury become sensitised (Latremoliere and Woolf 2010).

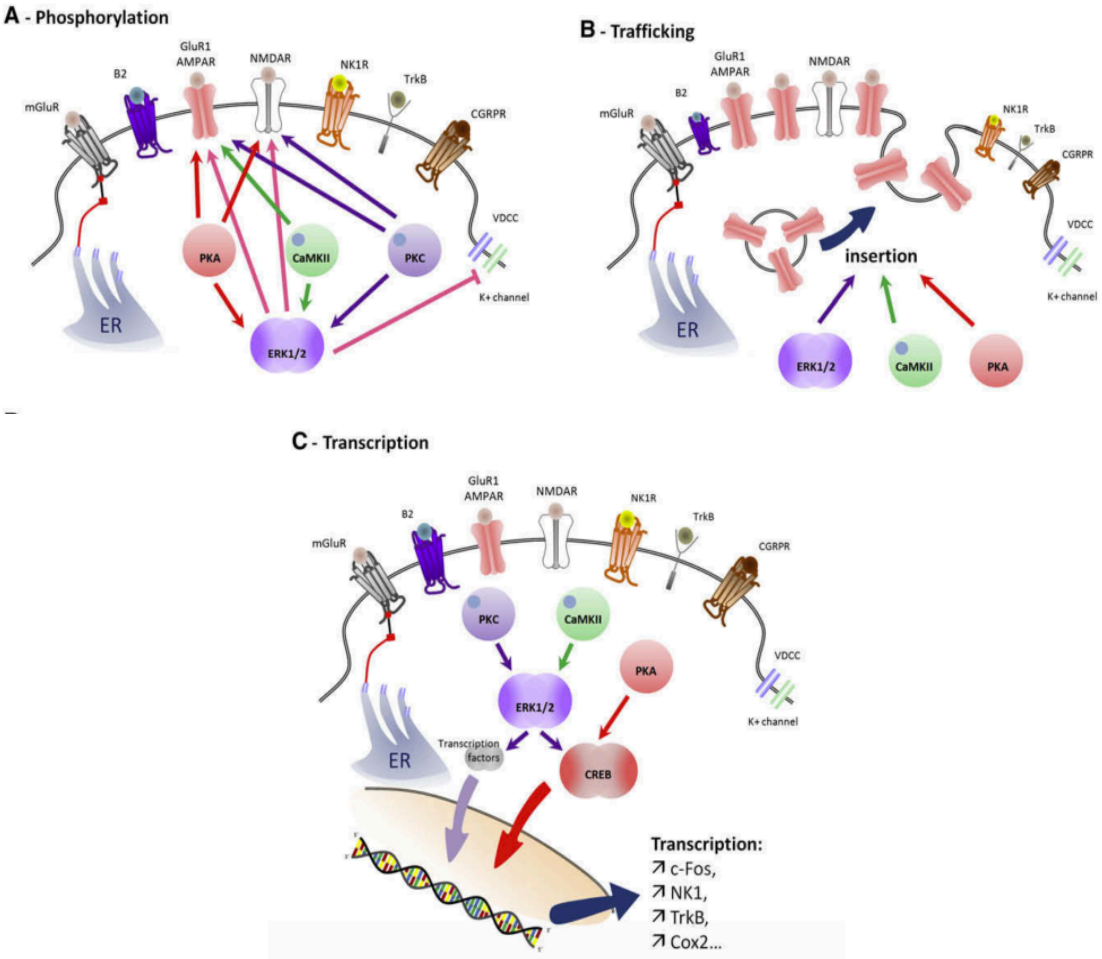


Figure 1.7. The intracellular pathways responsible for activity dependent central sensitisation. The influx of calcium through NMDA receptors and activation of TrkB, CGRP and NK1 receptors cause activation of intracellular protein kinases. (A)PKC, PKA and CaMKII phosphorylation of post-synaptic receptors alters their threshold and activation kinetics to produce an increased synaptic efficacy. (B).This phosphorylation can also result in increased trafficking of these receptors to the plasma membrane. (C) Transcirptional changes can be mediated by CREB, driving the expression of pain responsive genes: c-Fos, neurokinin 1 (NK1), tyrosine kinase B (TrkB) and cyclooxygenase-2 (Cox-2). mGluR – metabotropic glutamate receptor, B2 – β2 adrenoceptor, AMPAR - α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid Receptor, NMDAR - N-methyl D-aspartate Receptor, NK1R – Neurokinin-1 receptor, TrkB – Tyrosine kinase B receptor, CGRPR – calcitonin gene-related peptide receptor, VDGC – voltage-dependent gated channel, ER – endoplasmic reticulum, PKA – protein kinase A, CaMKII - Ca^{2+}-calmodulin-dependent protein kinase II, PKC – protein kinase , CREB – cAMP response binding element protein. Figure taken from (Woolf and Salter 2000).

The overall and combined effect of central sensitisation mechanisms is an amplified response to subsequent noxious and innocuous stimulation (McMahon et al 2013, Section 1 Chapter 5). Peripheral sensitisation and central sensitisation often exist together in chronic pain states. For instance, the barrage of peripheral nociceptive information due to peripheral sensitisation can lead to the development of central sensitisation. Therefore, it can be difficult to dissect the specific peripheral and central contributions resulting in an increased pain perception.

1.1.6.1 Glia in the spinal cord

Within the central nervous system, glial cells outnumber neurons ten to one (McMahon et al 2013, Section 1 Chapter 5). Glial cells are not directly involved in synaptic signaling of electrical activity but provide functional support to the neurons by maintaining their synaptic connections and signaling ability, glia are abundant in the spinal cord and are believed to play a crucial role in central sensitisation (Purves et al 2001). The neurons can influence the functional properties of glial cells, and vice versa, neurons can initiate signals to alter the function of glial cells, this causes the glial cells to release factors that regulate neuronal function (McMahon and Malcangio 2009). There are three types of glial cells found in the central nervous system: the oligodendrocytes, astrocytes and microglia. In the central nervous system, oligodendrocytes are involved in providing support and insulation to the neurons through myelinating axons (Purves et al 2001).

Astrocytes are the most abundant glial cell type in the central nervous system making up around 40-50% of the glial cells. Astrocytes are derived from the neuroectoderm and can become activated in response to tissue damage (Milligan and Watkins 2009). In inflammatory pain models, both astrocytes and microglia are activated in the spinal cord (Bradesi 2010). The astrocytes have an intimate relationship with neurons as they enwrap synapses to provide anatomical support. Furthermore, astrocytes also regulate the extracellular ions in the synapse through the use of voltage gates channels on their cell surface. There are two mechanisms by which astrocytes can regulate extracellular ions. Firstly, astrocytes express a Na^+/K^+ ATPase channel, which is responsible for the removal of K^+ from the synapse. Secondly, astrocytes can remove glutamate from the synaptic cleft via high affinity glutamate transporters expressed on their cell surface. Through these mechanisms astrocytes help to maintain a normal synaptic excitability but following nerve injury the expression of these channels can be differentially regulated, the subsequent deficient removal of glutamate and K^+ from the synapse could contribute to the enhancement of neuronal excitability (Xin et al 2009).

Microglia are a population of macrophages with a mesodermal origin, being derived from myeloid precursor cells. In the normal setting, microglia are dormant but upon injury or tissue damage they can detect various stimuli or factors that may be released into the spinal cord that may threaten the physiological homeostasis (McMahon and Malcangio 2009). Upon detecting tissue damage associated stimuli microgliosis can occur, whereby the local density of microglia increases by local proliferation and

through migration of these cells from other sites within the central nervous system (Calvo and Bennett 2010). Upon detection of potentially harmful stimuli and activation of the microglia, they change their morphology from a resting ramified shape into an active amoeboid one. Activation of microglia can also occur due to damage outside of the central nervous system; when peripheral afferents become damaged microgliosis occurs in the dorsal horn where the injured sensory afferents terminate. Activated microglia can exacerbate the pain state further through the production of pro-inflammatory cytokines, chemokines and extracellular proteases (Schomberg and Olsen 2012).

The activated microglia express ATP-gated P2X receptor ion channels on their cell surfaces, which are permeable to Ca^{2+}. ATP can be released by activated primary afferents, glial cells, and central neurons (McMahon and Malcangio 2009). When ATP stimulates these receptors, a Ca^{2+} influx occurs in microglia and subsequently activates downstream signaling proteins. One such pathway that becomes activated is the p38 mitogen-activated protein kinase (p38 MAPK), which can subsequently cause release of the cysteine protease Cathepsin S (CatS) from microglia (Clark and Malcangio 2012). The release of CatS can liberate the transmembrane chemokine Fractalkine (FKN) from neurons, which can then feed back to microglia to promote activation of the p38 MAPK pathway and enhance the release of inflammatory mediators further (Clark and Malcangio 2012). This creates a positive feedback loop between the neurons and microglia that ultimately causes an increase in the activation of neurons signaling to higher centres in the brain. Therefore, pharmacologically interrupting the cathepsin/fractalkine signaling could prevent hypersensitivity developing in chronic pain states. Previous studies have confirmed this, firstly a pharmacological inhibitor of p38 MAPK has been shown to reverse mechanical allodynia (Tsuda et al 2003). Secondly, the expression of the $P2X_4$ receptor has been found to be increased in the ipsilateral dorsal horn in response to nerve injury. The pharmocological blockade of this receptor with an intrathecal antagonsist or intraspinal administration of $P2X_4R$ antisense oligodeoxynucleotides prevented allodynia in a spinal nerve ligation model (Tsuda et al 2003). Overall, the ATP-mediated communication between neurons and microglia in the spinal cord may be a major mechanism responsible for exacerbation and development of central sensitisation.

Additionally, the activation of glia can induce the expression of pro-inflammatory cytokines in the spinal cord. A spinal injection of the pro-inflammatory cytokines IL-1β, TNFα and IL-6 increase CREB phosphorylation in superficial dorsal horn neurons and

induce hyperalgesia (Kawasaki et al 2008). Furthermore, TNFα and IL-1β enhance AMPA and NMDA induced currents, while IL-1β and IL-6 suppress GABA and glycine induced currents. Therefore, pro-inflammatory cytokines can induce synaptic and contribute to central sensitisation by increasing excitatory and/or decreasing inhibitory synaptic transmission (Kawasaki et al 2008).

1.1.7 Ascending projections, the brain and descending controls

Once the nociceptive information in the periphery has converged onto neurons in the dorsal horn, projection neurons can form ascending tracts that transmit the nociceptive signal to specific brain regions. Upon reaching higher centers in the brain, the nociceptive information can be consciously perceived as a pain signal. Multiple brain regions are responsible for the processing of the pain signal and may subsequently project from the site from which descending fibres are projected back to the dorsal horn of the spinal cord. These descending fibres can have either inhibitory or facilitatory actions, which modulate pain processing by determining the overall excitability of dorsal horn neurons. Therefore, the descending controls are largely responsible for determining pain perception as a result of peripheral nociceptive drive.

1.1.7.1 The spinothalamic tract

A main ascending pathway involved in transmitting nociceptive activity from the dorsal horn of the spinal cord to the brain in humans is the spinothalamic (STT) tract. This pathway initiates in the spinal grey matter, from where it can convey pain-related information directly to the thalamus. This pathway is associated with the sensation of pain, itch and temperature and can originate from lamina I or IV-V cells within the dorsal horn (McMahon et al 2013, Section 1 Chapter 11). The thalamus is a supraspinal structure responsible for the processing and integration of nociceptive signals before transferring this information on to other brain structures. It is the role of the thalamic relay nuclei to filter and process sensory information before it is sent to the cerebral cortex (Kuner 2010). It can encode information regarding the type, temporal pattern, intensity and localization of the pain signal, whilst interlinking with cortical and limbic structures involved in both the sensory-discriminative and emotional components of the pain sensation (Milligan 1998).

1.1.7.2 The spino-bulbo-spinal loop

There are also indirect ascending pathways that send messages to the forebrain through the contact of homeostatic sites within the brainstem, these are the spinobulbar projections. It was shown using retrograde labeling that spinobulbar cells initiating these tracts are found specifically in lamina I, V and VII in primates (Wiberg et al 1987). Two major areas of the brainstem where these spinobulbar projections terminate are the parabrachial nucleus and the periaqueductal grey (PAG). The PAG receives direct spinal nocicieptive input and is connected with other levels of the central nervous system including the forebrain structures involved in the transmission of pain . This makes it ideally located for integration of brain areas involved in the emotional-affective components of pain (Cerminara et al 2009). Therefore, the PAG is thought to be a critical site for managing different types of stress and threat (Vaccarino et al 1997). Similar to the spinobulbar projections are the spinomedullar projections, which contact homeostatic sites in the medulla of the brain but also have further links to forebrain areas (McMahon et al 2013, Section 1 Chapter 11).

Importantly, this system is a loop and therefore it also forms projections from the brain back to the spinal cord where dorsal horn excitability is modulated. The PAG is an important site for the initiation of descending pathways, which project to the dorsal horn via the rotralventomedial medulla (RVM) The Rostroventromedial Medulla (RVM) is a heterogenous area within the brainstem that consists of several nuclei including the 5HT rich Nucleus Raphe Magnus (NRM). It receives direct sensory input whilst also relaying descending modulatory projections to both superficial and deeper dorsal horn neurones of the spinal cord (Milligan 2002, Suzuki et al 2004, Bee and Dickenson 2007, Heinricher et al 2009). The RVM is the final relay for midbrain modulatory influences and can facilitate or inhibit incoming inputs to the deep dorsal horn. (Fields et al 1991). The PAG-RVM system can inhibit spinal nociceptive transmission, preferentially inhibiting C-fibre mediated nociceptive signals (Heinricher et al 2009). Peripheral tissue damage can have long-lasting effects on the excitability of cells found in the RVM (Gebhart 2004).

The RVM is capable of bimodal modulation through recruitment of different cell types, which can be characterized by their responses to noxious stimuli. OFF-cells are inhibitory and under normal conditions they are constantly firing. However, in response

to a noxious stimulus OFF cells will pause in activity immediately before a reflex (De Felice et al 2011). GABAergic axons arising in the RVM arborize throughout the dorsal horn and can form synapses with lamina II interneurons, indicating a mechanism for the RVM mediated inhibition in the dorsal horn. On the contrary, ON-cells will accelerate in firing in response to a noxious stimulus before a nociceptive reflex (Porecca 2002). It is these cells that are responsible for the descending facilitation from the RVM and are inhibited by analgesic opioids.

1.1.7.3 The cortical processing of pain

There are two different types of neuroanatomical ascending pathway; monosynaptic pathways project directly to higher cerebral structures while polysynaptic pathways use a relay station with other neurones to transmit the information (Milligan 1998). The ascending pathways that carry nociceptive information are complex and stimulate many areas within the brain. The areas of the brain consistently activated by nociceptive input are known as the "pain matrix" as shown in Figure 1.8. Through the use of imagining techniques in humans it has been possible to detect these regions. The identified areas of the brain are the anterior cingulate cortex (ACC), the primary and secondary somatosensory cortices, the prefrontal cortices and the thalamus (Tracey and Manyth 2007)(Talbot et al 1991). Additionally, the hypothalamus and amygdala are activated in the pain process through projections from the parabrachial nucleus, and these are known as the spinohypothalamic and spinoamygdalar tracts. The involvement of these brain areas in nociceptive transmission would explain why pain is accompanied by motivational-affective symptoms like anxiety and suffering (Benzon et al 2013)(Rainville 2002). Overall, the pain matrix has two components; the sensory-discriminative component determines the location and intensity of the incoming pain signal while the affective-cognitive component defines the emotional aspect of the pain experience (Tracey and Mantyh 2007).

1.1.7.4 Descending pathways

Cortical centres can influence spinal function through monoaminergic descending pathways (Bannister et al 2009). The serotonergic (5-HT) descending pathway originates mainly in the nucleus raphe magnus of the RVM while the noradrenergic (NA) descending system arises from the locus coeruleus (LC) and other pontine regions of the brainstem. The axons originating from these brainstem areas terminate diffusely throughout the dorsal horn. While some of these axons form synapse in the dorsal horn,

much of the action of 5-HT and NA is mediated through volume transmission (Todd 2010).

Once the monoamines are released into the dorsal horn, they can have inhibitory or faclitatory actions depending on the receptor they activate. Noradrenaline has inhibitory actions in the spinal cord through activation of α_2-adrenergic receptors. The α2-adrenergic receptors are found on both the central terminals of primary afferent fibres where NA elicits pre-synaptic inhibition, and on spinal pain-relay neurons where NA mediates post-synaptic inhibition. Noradrenaline can also mediate inhibitory actions in the spinal cord through its binding to α_1-adrenergic receptors on inhibitory interneurons (Petrovaara 2013).

The actions of 5-HT are more complex and have been shown to mediate both pro-nociceptive and anti-nociceptive actions at the level of the spinal cord. 5-HT can mediate facilitatory actions in the dorsal horn, particularly in chronic pain states, as the depletion of spinal 5-HT attenuates hypersentivity in neuropathic pain (Rahman 2006). Both 5-HT_2 and 5-HT_3 receptors are shown to play pro-nociceptive roles in the dorsal horn, implicating them in the facilitation carried out by 5-HT (Rahman et al 2011, Suzuki et al 2002). However, the spinal application of 5-HT has an overall antinociceptive role indicating that activation of other 5-HT receptors in the spinal cord mediate inhibition (Milligan 1995). Recently, the 5-HT_7 receptor has attracted attention as a promising candidate for carrying out the antinociceptive actions of 5-HT. Indeed, the analgesic action of morphine microinjected into the RVM is prevented with the spinal application of a 5-HT_7 receptor antagonist (Dogurl et al 2009). This suggests that inhibitory descending pathways arising in the RVM act in the spinal cord through activation of the 5-HT_7 receptor.

A balance exists between the descending inhibitory and facilitatory pathways. However, the plasticity that exists within these NA and 5-HT pathways suggests they can be altered in chronic pain states. Specifically, an increased 5-HT facilitatory drive and a downregulation of the NA descending pathway may contribute to the maintenance of hypersensitivity in chronic pain (Porecca et al 2002, Leiphart et al 2003).

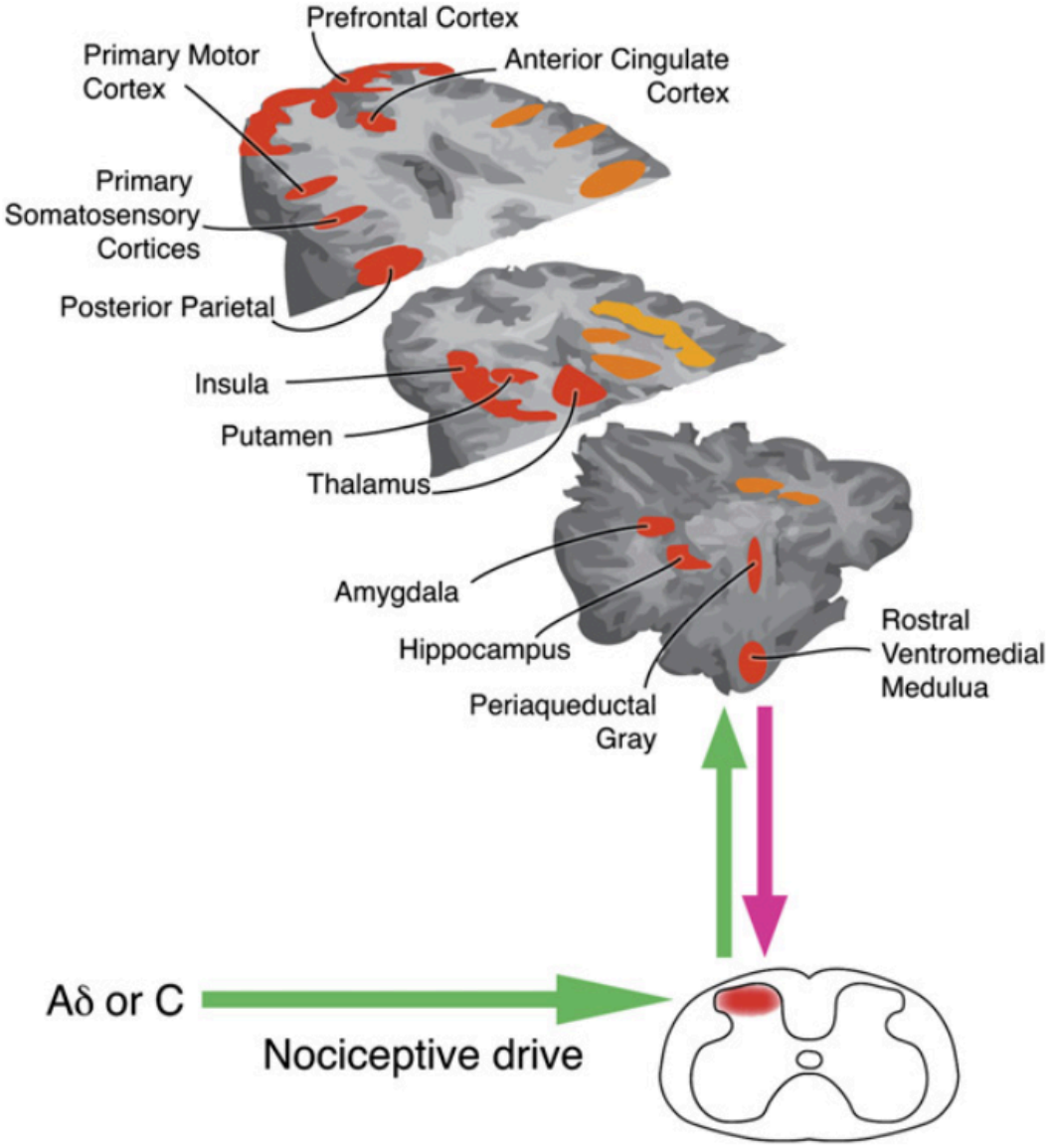


Figure 1.8. The brain regions involved in the processing of the pain signal. Peripheral afferent fibres carry nociceptive information to the dorsal horn of the spinal cord. Projection neurons then carry the nociceptive signal from the dorsal horn to higher brain centres along ascending pathways. The highlighted areas show the brainstem, midbrain and forebrain areas activated during the pain experience. Descending controls can then modulate the pain signal at the level of the spinal cord by exhibiting a facilitatory or inhibitory drive.

1.2 Diffuse Noxious Inhibitory Controls

1.2.1 What are diffuse noxious inhibitory controls?

Diffuse noxious inhibitory controls (DNIC) describe the phenomenon whereby one pain inhibits another. This unique form of endogenous inhibitory control can be seen in spinal and trigeminal convergent WDR neurons (Le Bars 1979a, Le Bars et al 1981, Cadden et al 1993). All activities of WDR neurons, both noxious and innocuous, can be strongly inhibited by a second concomitant noxious conditioning stimulus outside of their receptive field (Bannister and Dickenson 2017). The WDR neurons are important for the transmission of nociceptive signals through the dorsal horn. The inhibition of the transmission of these signals, mediated by a nociceptive stimulation that is outside of their own receptive field, demonstrates that this inhibitory modulation is not somatotopically organized but rather applies to the whole body (Le Bars and Willer 2008). An intense noxious conditioning stimulus can cause long-lasting post-stimulus effects, where persistent inhibition of convergent neurons can be seen for several minutes following removal of the conditioning stimulus (Le Bars and Willer 2008). This phenomenon is often referred to as counter irritation or counter stimulation.

DNIC is mediated by a final post-synaptic mechanism that acts on the cell bodies of convergent neurons in the spinal cord (Villanueva et al 1984). To confirm this extracellular recordings were taken from convergent neurons in the dorsal horn that were excited by electrophoretic application of glutamate, and this was then repeated in the presence of a noxious conditioning stimulus (Villanueva et al 1984). The doses of glutamate used were much greater than the threshold for firing and when applied alone induced an intense discharge and excessive depolarization of the convergent neurons. Following application of a concomitant noxious conditioning stimulus, the glutamate-evoked activity in convergent neurons was significantly depressed. This demonstrates that despite the presence of glutamate in the spinal cord, as would be the case with activation of peripheral afferent fibres terminating centrally in the dorsal horn, the activation of second order neurons is prevented. This suggests that the conditioning noxious stimulus triggers inhibitory post-synaptic potentials and hyperpolarization of the membrane of spinal convergent neurons (Villaneuva et al 1984a, Villaneuva et al 1984b).

To elicit DNIC the conditioning stimulus must be noxious, suggesting that DNIC is specifically triggered by the activation of peripheral Aδ and C nociceptors. The fibre types required for DNIC expression were investigated in rats using recordings from trigeminal WDR neurons that were excited by microelectrophoretic application of glutamate (Bouhassira et al 1987). The percutaneous application of single square-wave electrical stimulus to the tail induced a biphasic depression of trigemincal WDR neuron activity. The early and late components of this inhibition occurred with shorter latencies when the base of the tail was electrically stimulated, compared to the tip. The differences in the latencies were used to calculate the conduction velocities of the peripheral fibres triggering the biphasic inhibition. The mean conduction velocities were found to be $7.3ms^{-1}$ and $0.7ms^{-1}$, which fall into the Aδ and C fibre ranges respectively (Bouhassira et al 1987). This suggests that DNIC is made up of two inhibitory components, one is mediated by the activation of Aδ fibres and the other component is mediated by the activation of C fibres.

The induction of inhibitory post-synpatic potentials in convergent spinal neurons requires the activation of supraspinal structures and functional descending controls. DNIC cannot be observed in convergent neurons in anesthetized animals with spinal cord transection (Le Bars et al 1979b, Dickenson and Le Bars 1983). This confirms that the endogenous inhibitory controls utilized by DNIC are not confined to the spinal cord and require supraspinal structures. It has been proposed that the conditioning noxious stimulus activates supraspinal structures, and this subsequently initiates descending inhibitory controls that act upon neurons in the dorsal horn to mediate the inhibitory response.

1.2.2 What is Conditioned Pain Modulation?

The phenomenon of one pain inhibiting another pain can also be observed in humans, where the mechanism is known as Conditioned Pain Modulation (CPM)(Nir and Yarnitsky 2015, Yarnitsky 2010). CPM can be observed in the clinic when a distant painful conditioning stimulus is used to affect a test stimulus. Similarly to DNIC, the second conditioning noxious stimulus must be noxious. CPM can be initiated by noxiously stimulating multiple areas of the body, but this noxious stimulus must be outside of the excitatory receptive field of the original stimulation. This whole body effect suggests that like DNIC, CPM is mediated by a final post-synaptic mechanism in the spinal cord.

Similarly to DNIC, CPM requires supraspinal structures and intact descending pathways as demonstrated by its absence in tetraplegic patients (Roby-Brami et al 1987). This was confirmed in a study where the nociceptive spinal flexion reflex RIII was evoked in the right leg by electrical stimulation of the sural nerve, and a noxious conditioning stimulus was applied by electrical stimulation on the left hand. Responses in normal human subjects were compared to tetraplegic patients with lesions in the C5-C7 levels of the spinal cord due to traumatic injury. In normal subjects, strong depression of the spinal flexion reflex and the associated pain were seen in the presence of the conditioning stimulus. However, in tetraplegic patients the noxious conditioning stimulus did not produce any depression of the spinal flexion reflex (Roby-Brami et al 1987). The conditioning stimulus used was electrical stimulation applied to the digital branches of the ulnar nerve, which was below the level of spinal transection for all patients. This suggests that the inhibitory effects on the RIII reflex mediated by a conditioning nociceptive stimulus are sustained by a loop that requires the activation of supraspinal structures.

The similarities observed in the function of DNIC and CPM offers opportunities for forward translation from preclinical studies to the clinic (Bannister and Dickenson 2016).

1.2.3 DNIC in chronic pain

The transition from an acute to chronic pain state often involves an altered function of endogenous pain modulation (Porecca 2002, Rahman 2006, Bannister et al 2015). Analysing the underlying mechanisms of DNIC or CPM provides a method for investigating the descending modulation of pain (van Wijk and Veldhuizen 2010). In a wide and varied array of chronic pain conditions, there is a less efficient or abolished DNIC. The facilitation and inhibition of nociception occurs simultaneously, mediated through descending controls originating in the brainstem. The noxious conditioning stimulus induces DNIC via activation of the descending systems. Therefore, DNIC studies measure the net output. As such, a lack of DNIC could be due to a combination of enhanced facilitatory influences and a masked inhibitory controls (Arendt-Nielsen et al 2008).

Compromised DNIC has been shown in both human studies and animal models of osteoarthritis. This was demonstrated in a rat model by comparing acute and chronic

monoarthritis induced by injection of CFA into the ankle joint. Behaviourally, both groups displayed an increased sensitivity to mechanical stimuli applied to the ankle. Electrophysiological recordings taken from trigeminal convergent neurons in the animals with acute monoarthitis displayed a large inhibition upon application of a concomitant mechanical or thermal noxious conditioning stimulation at the injured joint. In contrast, this large inhibitory response was lost from trigeminal convergent neurons in animals with chronic monoarthritis, the authors suggested that the inputs activated by the conditioning noxious stimulus on the injured joint could no longer recruit a DNIC response (Danziger 1999). Similar results have been found in a human study, where pain pressure thresholds (PPTs) were measured around the knee joint in patients with both severe and moderate osteoarthritis of the knee. The PPTs were then reassessed in the presence of cuff pressure stimulation on the arm as the noxious conditioning stimulus. The CPM responses were compared to healthy age-matched controls. In patients with both severe and moderate osteoarthritis of the knee, there was no increase in PPT indicating a lack of CPM (Arendt-Nielsen et al 2010).

A dysfunctional CPM is also demonstrated in humans with experimentally induced muscle pain. Firstly, PPTs around the knee joint were measured in healthy human subjects. A cold pressor was then applied as a noxious conditioning stimulus, and this produced an increase in PPTs demonstrating CPM. However, when the subjects were injected with hypertonic saline to evoke muscle pain the increases in PPT due to the cold pressor conditioning stimulus were much smaller. This suggests that patients with chronic musculoskeletal pain may have impaired CPM (Arendt-Nielsen et al 2008).

Fibromyalgia patients present with chronic persistent pain, yet no damage or inflammation in the nervous system can be detected. A CPM paradigm in the clinic was used to analyse the function of descending modulation in fibromyalgia patients (Lautenbacher and Rollman 1997). Electrical pain thresholds were determined and used as the test stimulus, while a tonic thermal stimulation was used as the conditioning stimulus. The concurrent tonic thermal stimuli significantly increased the electrical pain threshold in pain-free control subjects but not in fibromyalgia patients (Lautenbacher and Rollman 1997). Interestingly, it was also found that fibromyalgia patients with depressive symptoms had increased clinical pain and a more pronounced deficit in CPM than fibromyalgia patients lacking these symptoms (de Souza et al 2009). Depression is often associated with chronic pain and both have been reported to involve

dysnfunctional descending controls, indicating that fibromyalgia pain could arise from an imbalance in descending inhibitory and facilitatory pathways.

A further chronic pain state with abnormal endogenous inhibitory mechanisms is irritable bowel syndrome (IBS). In both IBS patients and normal subjects, rectal pain was scored during rectal balloon distention alone or in the presence of noxious heterotopic stimulation of the foot in ice water. In the healthy controls, the conditioning stimulation decreased rectal pain but this was not the case in IBS patients (Wilder-Smith et al 2004).

Furthermore, through assessing the functionality of a patients CPM expression before surgery, it can be predicted how likely the patient is to develop post-operative chronic pain. DNIC assessment was conducted in 62 patients before and after thoracotomy. The efficiency of pre-operative CPM was negatively correlated with chronic post-operative pain, indicating that a weak CPM before surgery may predict a patient's susceptibility to developing chronic pain. This suggests that the endogenous inhibitory system activated by CPM is required for coping with noxious events. Assessing this in patients could allow individually tailored pain prevention and post-operative management (Yarnitsky et al 2008).

Interestingly, the abolished DNIC/CPM system often found in chronic pain states can be restored upon patient recovery or through pharmacological manipulation. In cluster headache patients a cold pressor was used as a conditioning noxious stimulus to inhibit a nociceptive withdrawal reflex. When the patients were undergoing the active phase of the headache, the cold pressor could induce no inhibitory effect on pain responses indicating a dysfunctional CPM. Yet, during the remission phase the cold pressor produced a clear inhibition, indicating that CPM was quickly restored (Perrotta et al 2013). Secondly, in patients with painful osteoarthritis of the hip, PPTs were not influenced by a heterotopic noxious conditioning stimulation. Following hip replacement surgery, the patients who were pain free were re-assessed, their PPTs were now significantly modulated by the heterotopic noxious conditioning stimulation (Kosek and Orderberg 2000). This suggests that the chronic nociceptive pain maintained the dysfunctional CPM as observed pre-surgery. Upon relief of the chronic persistent pain, the CPM system could reset itself to a functional state. Finally, it has recently been shown that DNIC is abolished in a spinal nerve ligation model of neuropathy in the rat (Bannister et al 2015). However, the authors showed they could pharmacologically

restore the expression of DNIC by manipulating the descending monoaminergic system (Bannister et al 2015). Taken together, these finding suggest that the loss of DNIC/CPM in chronic pain states is due to a plasticity in descending controls rather than a complete abolishment of this system.

1.2.4 Role of monoamines in DNIC

DNIC expression requires activity of supraspinal structures, functional descending controls, and is lost in multiple chronic pain conditions. As noradrenergic and serotonergic descending controls arise in the brainstem and are also modulated in chronic pain states, the monoamines seemed likely candidates for neurotransmitters involved in the final postsynaptic mechanism mediating DNIC in convergent neurons.

Serotonin transporter gene polymorphisms affect the magnitude of CPM in healthy subjects (Lindstedt et al 2011). The serotonin transporter (5-HTT) is an important component for 5-HT signaling as it ceases the actions of extracellular 5-HT through sodium-dependent intracellular reuptake (Le Bars 2002). Low expression of the 5-HTT gene has been associated with an increased risk of developing chronic pain, and polymorphisms of the 5-HTT gene have been implicated in the development of fibromyalgia (Offenbaecher et al 1999). Lindstedt et al carried out a study in which healthy subjects were grouped according to their 5-HTT genotype. Pateints were spilit into either high or low expressing groups. Individual noxious heat and pain pressure thresholds (PPTs) were assessed and used as subjective test stimuli. A submaximal-effort tourniquet test was used as the noxious conditioning stimulus to evoke CPM. The authors found that the low 5-HTT expressing patient group exhibited a significantly lower degree of CPM-mediated inhibition for both pressure and heat pain (Lindstedt et al 2011). This indicates that the level of 5-HTT expression is an important contributing component in determining the efficiency of an individuals CPM.

Another human study that investigated the efficacy of duloxetine in patients with neuropathic pain strongly supports the concept that noradrenaline and serotonin mediate CPM (Yarnitsky et al 2012). Duloxetine is a serotonin noradrenaline reuptake inhibitor (SNRI). Pain modulation assessment was carried out before duloxetine treatment to measure individual baseline CPM efficacy. The authors found that baseline CPM was correlated with the efficacy of duloxetine, such that duloxetine was most effective in patients with a less efficient CPM (Yarnitsky et al 2012). This suggests that

patients with a malfunctioning pain modulation will benefit from drugs that restore the functionality of descending inhibitory pain control. Meanwhile, patients with a functional CPM may not benefit from such drugs as they already have a functional descending inhibition. Determining the type of drug to administer to patients by evaluating the functionality of their CPM promotes the development of personalized medicine.

It has been demonstrated in naïve rats that activation of the DNIC system requires the action of noradrenaline (NA) acting at α2-adrenoceptors in the spinal cord (Bannister et al 2015). When the α2-adrenoceptor antagonists yohimbine and atipamezole were administered in naïve animals DNIC expression was completely abolished. This demonstrates that a NA mediated mechanism acting via α2-adrenoceptors in the spinal cord, is crucial for the activation of DNIC. During the transition to a chronic pain state the descending monoaminergic pathways undergo dynamic changes, which can lead to an increased descending facilitatory drive and this is mediated by 5-HT, as well as a downregulation of the descending noradrenergic system (Suzuki et al 2004, Rahman et al 2008). In a spinal nerve ligation model of neuropathy in the rat, where the descending systems are known to be dysregulated, DNIC was abolished (Bannister et al 2015). DNIC could be restored in these animals when the levels of NA were augmented with the noradrenaline reuptake inhibitors (NRIs) Reboxetine and Tapentadol. DNIC could also be restored by blocking 5-HT mediated descending facilitations acting at spinal 5-HT_3 receptor with the 5-HT_3 receptor antagonist ondansetron (Bannister et al 2015). This suggests after nerve injury a shift in the balance of descending inhibitory and facilitatory pathways occurs, and that a balance of these opposing systems is required for the maintenance of DNIC. The result is a dominant 5-HT facilitatory descending drive acting at 5-HT_3 receptors, which now outweighs the noradrenergic inhibitory pathway activated by the conditioning stimulus.

The involvement of noradrenaline and serotonin in DNIC will be discussed in greater detail in Chapters 4 and 6.

1.2.5 Supraspinal pathways sub-serving DNIC

DNIC is known to be mediated by a final post-synaptic mechanism that relies on supraspinal structures and functional descending controls. However, the exact brain regions required for DNIC to be expressed have not been conclusively determined. As

DNIC is decreased or abolished in chronic pain conditions, defining the supraspinal networks responsible for mediating DNIC may allow us to better understand the neural mechanisms responsible for the maintenance of persistent pain.

The involvement of serotonin and opioids in the DNIC system highlighted a potential role for brainstem structures that utilize these systems, such as the RVM. It was an early animal study that first suggested DNIC relies partially on a descending serotonergic pathway (Dickenson et al 1981). The authors found that animals depleted of serotonin due to pre-treatment with p-chlorophenylalanine (pCPA) had a strongly reduced DNIC response. The DNIC system is also thought to rely partially on endogenous opiates since a systemic injection of naloxone reduced the neuronal inhibition observed alongside application of a noxious conditioning noxious stimulus (Le Bars et al 1981). These findings led to the hypothesis that the dorsolateral funiculus pathway would be involved in the induction of DNIC as this comprises the PAG and RVM descending inhibitory projections onto the dorsal horn (Basbaum and Fields 1979). However, in animals with lesions of the PAG or RVM, convergent neurons display the same level of inhibition evoked by a conditioning noxious stimulus as compared to sham groups (Bouhassira et al 1990, Bouhassira et al 1993). Furthermore in human CPM studies, no signal intensity changes could be observed with fMRI in the PAG or medullary raphe when a noxious conditioning stimulus was applied (Youssef et al 2015). However, microinjection of morphine into the caudal PAG depressed the DNIC response (Dickenson and Le Bars 1987) and inactivation of the RVM with lidocaine restored DNIC in chronic morphine treated animals (Okada-Ogawa et al 2009). This suggests that the PAG and RVM may be able to indirectly modulate DNIC expression.

In humans CPM was investigated in patients with unilateral thalamic vascular lesions and also in patients with Wallenberg's syndrome, in which unilateral lesions of the retro-olivary portion of the medulla have occurred due to stroke (De Broucker et al 1990). In the patients with thalamic lesions, the RIII reflex was significantly inhibited by a nociceptive electrical conditioning stimulus applied to the hand. The nociceptive conditioning stimulus had long-lasting inhibitory effects that were comparable to that seen in healthy subjects (De Broucker et al 1990). The authors concluded that lemniscal and spinothalamic pathways were not required to trigger a DNIC response. In contrast, the patients with Wallenberg's syndrome displayed no inhibition of the RIII reflex with a conditioning noxious stimulus (De Broucker et al 1990). This suggested that the

brainstem and spinoreticular tract are vital components of the supraspinal loop that activates the DNIC response.

The spinorecticular tract contains branches that terminate in the most caudal part of the medulla, which suggests this component of the supra-spinal loop sub-serves DNIC (Bouhassira et al 1992a). Furthermore, a particular nucleus within this area, the subnucleus reticularis dorsalis (SRD), contains neurons that encode nociceptive information and respond specifically to activation of peripheral Aδ and C fibres from any part of the body, indicating that these neurons have a whole-body receptive field (Villanueva et al 1988). This theory was confirmed in rodents with unilateral quinolinic acid induced lesions of the SRD. In the SRD lesioned animals, neuronal inhibitions produced by heterotopic noxious conditioning stimuli were significantly reduced in spinal convergent neurons, suggesting that the SRD is a crucial supraspinal site in the neuronal circuitry mediating DNIC (Bouhassira et al 1992a). Despite the significantly reduced magnitude, DNIC mediated inhibition of spinal convergent neurons still remained in animals with SRD lesions. Additionally, low doses of systemic morphine have been shown to significantly depress the DNIC response, yet this has no effect on SRD neurons (Le Bars and Villanueva 1988, Bing et al 1989). This suggests that while the SRD is a vital brainstem structure in the expression of DNIC, other supraspinal structures may be involved in this endogenous inhibitory system.

Interestingly, it may be a reduction of activity within the SRD that is responsible for mediating a DNIC response. This was suggested following a human study that used fMRI to analyse brain structures involved in CPM analgesia in healthy subjects (Youssef et al 2015). Even in healthy individuals, only half of the subjects displayed a functional CPM indicating the variability of CPMs expression from one individual to the next. The authors reported that subjects with a functional CPM showed a reduction in signal intensity in the SRD when a test stimulus was applied in the presence of a noxious conditioning stimulus (Youssef et al 2015). Meanwhile, the changes in signal intensity in the SRD were not observed in subjects with no CPM response. The SRD is proposed to play a facilitatory role in pain processing; the activation of this nucleus through injection of glutamate causes a long-lasting enhancement of WDR neuronal responses to electrical stimulation of the sciatic nerve (Dugast et al 2003). The lack of CPM response observed in half the subjects could be caused by ongoing activity in the SRD. This suggests that the noxious conditioning stimulus mediates CPM through reducing neuronal activity within

the SRD and subsequently prevents the SRDs from having a facilitatory influence on convergent neurons in the dorsal horn.

The Parabrachial nucleus (PbN) is found in the brainstem and can influence the descending modulation of pain (Bernard et al 1996, Suzuki et al 2002). Interestingly, in the fMRI study discussed above the authors found a significantly reduced signal intensity in the Pb nucleus in subjects that displayed a functional CPM response upon application of a concomitant noxious conditioning stimulus (Youseff et al 2015). This contrasts with previous findings that show an ibotenic acid lesion of the PbN in the rat had no effect on the magnitude of inhibition seen in convergent trigeminal neurons upon application of a heterotopic noxious stimulus, which lead to the conclusion that this brain region was not critical for the DNIC response (Bouhassira et al 1990). These conflicting results suggest that the PbN is not directly involved in the supraspinal pathway sub-serving DNIC but is capable of modulating the DNIC response.

Higher brain structures in the cortex have also been demonstrated to modulate CPM expression (Youssef et al 2016). A further fMRI study found that subjects with no CPM response had signal intensity increases in the dorsolateral prefrontal cortex (dlPFC), the cingulate cortex (CC), and the amygdala upon application of a noxious conditioning stimulus. However, in individuals with a functional CPM, the signal intensity in these higher brain structures remained stable (Youssef et al 2016). Furthermore, in the subjects with a lack of CPM, the strength of functional connectivity between the SRD and dlPFC and CC increased upon application of the noxious conditioning stimulus (Youssef et al 2016). This suggests the dlPFC and CC can have a direct influence on the SRD. Therefore a lack of CPM may arise due to increased cortical control from the dlPFC and CC acting upon the SRD, which prevents its reduced activation and allows the SRD to continue sending facilitaory influences to convergent spinal neurons.

As DNIC is mediated predominantly by a noradrenergic mechanism, the locus coeruleus (LC) is a further brainstem structure which seemed likely to be involved in the DNIC circuitry. The inhibitory effect on spinal convergent neurons induced by a concomintant noxious conditioning stimulus relies on a noradrenergic descending system and the LC contains noradrenergic projections that terminate in the dorsal horn of the spinal cord (Bannister et al 2015, Kwiat and Basbaum 1992). This was investigated in rats with ipsilateral, contralateral and bilateral lesions of the LC (Bouhassira et al 1992b). The authors found no significant difference in the magnitude of inhibition seen in spinal

convergent neurons in these animals, compared to controls animals, when a heterotopic noxious conditioning stimulus was applied. This suggests the LC is not directly involved in the supraspinal loop that mediates DNIC. However, as the action of noradrenaline is crucial for a DNIC response, as shown by the complete abolishment of DNIC in naïve animals with α_2-adrenoceptor antagonists, it has been proposed there may be a projection from the SRD to the LC (Bannister et al 2015, Bannister et al 2017). If this is the case, reduced activity in the SRD triggered by a conditioning noxious stimulus may allow a noradrenergic pathway arising in the LC to become active. Therefore, DNIC may be triggered by a reduced facilitatory drive from the SRD and an increased inhibitory drive from the LC, which mediates the final inhibitory post-synaptic mechanism acting on convergent spinal neurons.

The SRD is a crucial brainstem nucleus required for the expression of DNIC (Bouhassira et al 1992a). Upon application of a heterotopic noxious conditioning stimulation a reduced activity in the region of the SRD reduces the facilitatory influences that arise in this brainstem region acting on convergent neurons in the dorsal horn (Youssef et al 2015). A reduction in the activity of the SRD may also allow the activation of a noradrenergic inhibitory descending system that arises from the LC (Bannister et al 2017). The activity of the SRD can be modulated by higher brain structures such as the dlPFC, CC and amygdala. Increased activity in these brain regions can act directly on the SRD to prevent the reduction in its activity and this causes a reduction in the magnitude of inhibition seen with DNIC (Youssef et al 2016). Despite lesion experiments showing the PAG and RVM are not involved directly in the surpapinal loop that sub-serves DNIC (Bouhassira et al 1990,1993), they are likely involved in the activation of monoaminergic descending controls that mediate DNIC in the spinal cord. Ultimately, a series of complex interconnections between midbrain and brainstem structures that comprise the dorsolateral funiculus pathway likely play an indirect role on the SRD and impact upon the release of serotonin and noradrenalin in the dorsal horn to mediate DNIC. The supraspinal structures that are thought to play a role in DNIC are shown in Figure 1.9.

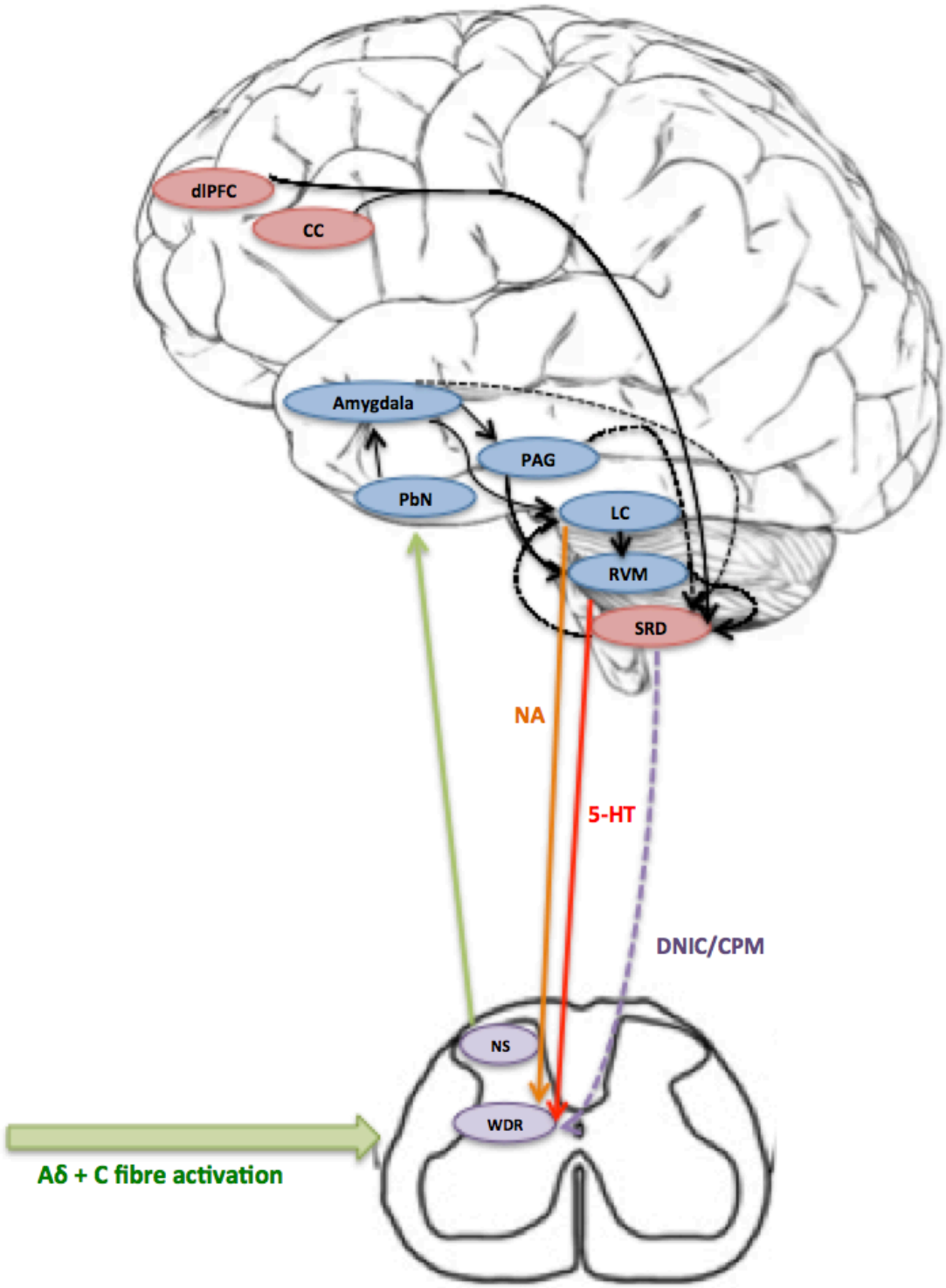


Figure 1.9. The supraspinal structures involved in the expression of DNIC. When Aδ and C fibres in the periphery are activated by noxious stimuli they project the signal onto second order nociceptive specific (NS) and wide-dynamic range (WDR) neurons in the dorsal horn of the spinal cord. Ascending projections then transmit the nociceptive signal to the parabrachial nucleus (PbN) which can then send further projections to structures in the limbic system such as the amygdala. Descending modulatory pathways can be triggered which arise in the Periaqueductal grey (PAG), rostraventral medial medulla (RVM), and Locus coeruleus (LC). This can inhibit or facilitate the responses of WDR neurons through the release of noradrenaline and/or serotonin. Upon application of a conditioning noxious stimulus, DNIC/CPM is mediated by the subnucleus reticularis dorsalis (SRD) in the caudal brainstem. The full block arrows show

the brain structures known to interact with the SRD. The dorsolateral prefrontal cortex (dlPFC) and cingulate cortex (CC) have been shown to have direct connections with the SRD and can modulate the expression of DNIC/CPM. The dashed arrows show the brain structures that potentially interact with the SRD and play a role in the modulation of DNIC/CPM. The reduced activity in the SRD induced by a conditioning noxious stimulus may act upon the LC to initiate noradrenergic descending inhibition. The RVM and PAG are not directly involved in the supraspinal loop subserving DNIC but may modulate DNIC expression through the activation of monoaminergic descending pathways that terminate in the dorsal horn.

1.3 Osteoarthritis

1.3.1 What is Osteoarthritis?

Osteoarthritis (OA) is a painful and disabling disease caused by a loss of function and integrity of the synovial joints. This disease affects a number of joints including the hands, feet, shoulders and spine but is most common in weight bearing joints such as the hips or knees. This thesis will focus on OA of the knee.

Structurally, OA can be characterized by the degenerative breakdown of articular cartilage, sclerosis of the subchondral bone, bony outgrowths known as osteophytes, and a thickened or inflamed synovium (McMahon et al 2013: Section 4, Chapter 43, Wieland et al 2005). Clinical symptoms of OA include joint stiffness and instability, functional impairment, movement-evoked pain, and episodic inflammation. Importantly, pain is the major clinical symptom of OA, and the reason patients report the disease (Peat et al 2001). Despite a clear clinical need for better OA pain management, current analgesics remain relatively ineffective.

OA can be triggered by a combination of mechanical, developmental, metabolic, and genetic factors that result in multiple pathophysiological mechanisms, but it remains difficult to pinpoint the exact causes for initiation and maintenance of the disease (Moskowitz 2007).

1.3.2 The subchondral joints

The joint can be described simply as the point at which two or more bones meet, they act to provide structural support and movement of the skeleton. Joints can be subcategorized into three categories based on their function and composition; these are fibrous, cartilaginous and subchondral joints. The synovial joints allow the most movement, and are the most common site of OA.

All subchondral joints are contained within a synovial capsule, which contains multiple strictures that aim to lubricate the joint and allow smooth movement. In the healthy subchondral joint, the articulating bone endings are not in direct contact due to a dense layer of articular cartilage covering the end of each bone. The cartilage is a firm visco-elastic tissue that acts as a cushion between the bones to prevent mechanical damage (Wieland et al 2005). The synovium is the inner membrane of the synovial capsule, it is a

highly innervated tissue and secretes synovial fluid to provide nutrients and lubrication to the joint. Finally muscles, tendons and ligaments surround the synovial joint to provide further structural stability.

Although the degradation of articular cartilage is a central feature of OA, it is becoming increasingly clear that OA is a disease of the whole joint (Loeser 2006, Dieppe 2011). The articular structures affected in OA of the knee are shown in Figure 1.10.

1.3.2.1 The Cartilage

The cartilage found in synovial joints is hyaline cartilage, this is a resilient tissue that reduces friction between bones and withstands high compressive loads to provide protection from mechanical forces. This is provided by the physico-chemical properties of the macromolecules that make up hyaline cartilage. Cartilage is composed of the highly differentiated mesenchymal cells known as chondrocytes. The chondrocytes are responsible for secreting and maintaining the extracellular matrix (ECM) components of the cartilage, while also controlling the movement of water and ions across the membrane (Muir 1995). The ECM components making up cartilage are collagen, proteoglycans, hyaluronic acid (HA), and a mixture of elastic fibres (Kiani et al 2002). Type II Collagen is the principle molecular component of articular cartilage in mammals, it is composed of three identical α chains that form a triple helix. The collagen molecules self-associate to form a fibrilar network, stabilized by intermolecular crosslinks, and this provides tensile strength to the ECM (Troeberg and Nagase 2012, Dejica et al 2008). The thin layer of calcified cartilage that provides the interface with articular cartilage and the bone also contains collagen X (Eyre 2002). Aggrecan is the major proteoglycan found in the articular cartilage, it is formed of numerous monomers non-covalently bound to HA (Kiani et al 2002, Roughley and Mort 2014). Aggrecan is responsible for drawing water into the matrix and allows the cartilage to resist compression (Troeberg and Nagase 2012). A dense network of collagen and aggrecan fibres is essential for the biochemical properties that maintain the mechanical protection provided by cartilage (Wieland et al 2005). The articular cartilage contains no nerves or blood vessels.

In osteoarthritis the anabolic/catabolic mechanisms that maintain the homeostasis within articular cartilage is disrupted, which can lead to striking alterations in the physical structure of articular cartilage (Dejica et al 2008). This is mediated by the chondrocytes; a number of genetic, biochemical, or mechanical stress factors can cause a

disruption in chondrocyte-matrix associations and an alteration of the chondrocytes metabolic responses. The early morphological changes associated with early OA are cartilage surface fibrillation and cleft formation. As the disease progresses there is an increased synthesis of catabolic cytokines in the chondrocytes of catabolic cytokines and ECM, leading to a loss of cartilage volume(Goldring 2000, Dieppe and Lohmander 2005).

One of the early events in the development of OA is the degradation of aggrecan. Metalloproteinases (MMPs), which are capable of degrading proteoglycans, including aggrecans, and have been isolated from human articular cartilage (Gunja-Smith et al 1989). However, this remains controversial as it was shown that the aggrecan fragments present in the synovial fluid of OA patients had not been cleaved at an MMP sensitive site suggesting the MMPs were not responsible for the degradation of aggrecan (Sandy et al 1992). The ADAMTS (A distintegrin and metalloproteinase with thrombospondin motifs) are zinc-dependent metalloproteinases and the major enzymes responsible for the pathological cleavage of aggrecan in OA (Sandy and Verscharen 2001). This has been particularly demonstrated with ADAMTS5, as knockout mice develop less severe cartilage damage in a destabilized medial meniscus model and an antigen-induced model of OA (Glasson et al 2005, Stanton et al 2005). Interestingly, ADAMTS4$^{-/-}$ knockout mice did not exhibit this protection against aggrecan degradation (Glasson et al 2004), but suppression of ADAMTS4 attenuated the degradation of aggrecan in human cartilage explants (Song et al 2007). Song et al also found a reduction in aggrecan degradation in human OA cartilage following siRNA mediated knockdown of the ADAMST4 gene (Song et al 2007). In both ADAMTS1$^{-/-}$ knockout mice and in human OA cartilage lacking expression of the ADAMTS1 gene there is no inhibition of aggrecan loss, suggesting this enzyme may not play a role in cartilage degradation (Little et al 2005, Song et al 2007). Both ADAMTS4 and ADAMTS5 appear to play a role in the structural damage of cartilage observed in human OA, making them attractive targets for therapy.

Aggrecan plays a protective role to prevent degradation of the type II collagen fibrils in cartilage, such that degradation of collagen only occurs once aggrecan is lost from the cartilage (Pratta et al 2003, Karsdal et al 2008). Collagen is a stable molecule, yet it can be degraded by MMPs and Cathepsin K (CatK) (Troeberg and Nagase 2012). Particularly, there is an increased expression of MMP-13 in human OA cartilage (Kevorkian et al 2004). Furthermore, the conditional overexpression of constitutively active human MMP-13 in murine cartilage resulted in spontaneous degradation of articular cartilage (Neuhold et al 2001), while MMP-13 knockout mice with surgically induced OA showed

an inhibited erosion of cartilage even once aggrecan was depleted (Little et al 2009). MMP-1 can also efficiently cleave type II collagen and is thought to play a role in the degradation of human cartilage. This presents the MMPs as potential therapeutic targets, but their translation potential was hampered as high homology inhibitors tend to lack specificity and cause toxic and musculo-skeletal side effects (Troeberg and Nagase 2012). However, a MMP-13 specific inhibitor has been generated and this shown to effectively prevent cartilage damage in a surgical rat model of OA without producing any musculoskeletal side effects (Johnson et al 2007). Cathepsin K (CatK) is a cysteine protease expressed by chondrocytes in the articular cartilage (Connor et al 2009). In cartilage explants taken from patients with OA, CatK mRNA levels and the number of CatK-containing chondrocytes were found to be increased relative to the severity of OA (Konttinen et al 2002). In addition, CatK inhibitors were found to reduce collagen breakdown in cartilage explants from OA patients (Dejica et al 2008). Increased levels of MMPs and CatK may act together to mediate the pathological degradation of collagen within the articular cartilage as seen in advanced OA, and inhibiting the activity of these proteinases may provide an effective way of preventing such damage.

Tissue inhibitors of metalloproteinases (TIMPs) are endogenous inhibitors that can inhibit the activity of MMPs and some members of the ADAMTS family. TIMP-3 is thought to be a key player in the inhibition of cartilage degradation as it can inhibit both MMPs and ADAMTS (Kashiwagi et al 2001). TIMP3 knockout mice develop increased cartilage degradation (Sahebjam et al 2007), while an injection of TIMP-3 in a rat meniscal tear model of OA blocked the degradation of cartilage (Black et al 2006). Furthermore reduced levels of TIMP-3 protein were found in human OA cartilage compared to normal cartilage, suggesting a lack of this enzyme may contribute to the development of OA (Morris et al 2010).

Excessive mechanical loading can drastically alter the homeostasis of cartilage. Joint injury caused by extreme force or overuse can initiate a cascade of responses that result in the breakdown of cartilage and the subsequent pain and compromised joint function seen in OA. In mechanically injured cartilage co-cultured with joint capsule tissue, there was increased expression of MMP1, MMP3, MMP13, and ADAMTS5 genes in the chondrocytes (Lee et al 2009). The alteration of the genetic expression of chondrocytes and the subsequent upregulation of proteinases indicates how important a factor mechanical loading can be for initiating cartilage damage.

Once mature cartilage has been formed after development, it can become damaged due to instance mechanical forces or in response to inflammation. This can occur due to dysregulated expression of aggrecanases, collagenases, and their respective inhibitors. Aggrecan degradation resulting in cartilage damage is fully reversible due to chondroyctes capacity to repair this structure (Karsdal et al 2008, Roughley and Mort 2014). However once the collagen becomes damaged or undergoes degradation, it seems the chondrocytes have little capacity for repairing cartilage architecture (Eyre 2002, Karsdal et al 2008).

1.3.2.2 The synovium

The synovial membrane lies between the joint capsule and the joint cavity. The synovium is highly vascularized, and through the secretion of synovial fluid it supplies the avascular articular cartilage with nutrients and oxygen whist also providing lubrication. As the synovium and articular cartilage are in close proximity and solutes can flow freely between the two, the damaged cartilage has an effect on the synovial membrane. In many OA cases the synovial membrane is inflamed and enlarged (Revell et al 1988, Krasnokutsky et al 2008).

The histological changes observed in the synovial membrane of OA patients include hyperplasia, a thickening of the lining layer of cells, increased vascularity and an infiltration of lymphocytes (Samuels et al 2008, Wenham and Conaghan 2010). The synovial enlargement and inflammation observed in OA is confined to areas adjacent to the pathologically damaged cartilage, this is in contrast to rheumatoid arthritis (RA) where there is a much larger extent of synovial inflammation (Krasnokutsky et al 2008, Sweeney and Firestein 2004, Wenham and Conaghan 2010).

While the synovium has the physiological responsibility of nourishing the chondrocytes via the synovial fluid, synovial cells also phagocytose the metabolites and products of matrix degradation produced during injury (Sellam and Berenbaum 2010). The cartilage breakdown products in the synovial fluid can provoke the metabolically active synoviocytes to release collegenases and other hydrolytic enzymes which can exacerbate the destruction of articular cartilage further (Krasnokutsky et al 2008). Indeed, knee synovial fluid from OA patients showed a persistent increase in the levels of MMP-1 and MMP-3, while synovial specimens taken from patients with rapidly destructive hip OA show high levels of MMP-1, MMP-3 and MMP-9 (Tchetverikov et al

2005, Masuhara et al 2002). While this confirms the synovium is capable of producing MMPs that degrade cartilage, cartilage itself produces most of these destructive enzymes which work in a paracrine and autocrine fashion (Samuels et al 2008). The production of proteolytic enzymes by the synovium amplifies the cartilage degradation, causing more cartilage breakdown products to be deposited in the synovial fluid, which then further potentiates synovial inflammation (Sellam and Berenbaum 2010). When cartilage degradation begins in OA, a viscious positive-feedback loop can persist between the synovium and articular cartilage, which initiates and maintains and perpetuates the pathogenesis of the disease.

The inflammatory component associated with OA is discussed further in Section 1.3.3.

1.3.2.3 The bone

The femur, tibia and patellar are the bones that meet to form the synovial knee joint. Bone is a dynamically mineralized tissue, in response to mechanical demand it can adapt its functional state and begin a remodeling process (Goldring 2009). The joint contains three types of mineralized tissue with different mechanical and physiological properties, the calcified cartilage, the subchondral plate, and the subchondral trabecular bone. Directly between the articular cartilage and before the subchondral bone is a layer of calcified cartilage, a histological tidemark indicates the interface between articular and calcified cartilage. Following the calcified cartilage is the subchondral bone plate, this section of the bone is not very porous or vascular but is capable of remodeling by changing its density. Directly beneath the subchondral bone plate is the subchondral trabecular bone (Burr 2004). The advancement of imagining techniques such as magnetic resonance imaging (MRI) have allowed a more accurate determination of the changes that occur in the bones of OA. The most prominent abnormalities detected are bone marrow necrosis, fibrosis, osteophyte formation, an abnormally remodeled trabeculae and sclerosis of the subarticular bone (Wieland 2005, Conaghan et al 2006).

The subchondral bone and articular cartilage are intimately connected, together they adapt to mechanical stress (Buckland-Wright 2004). One of the processes that mediate this is remodeling of the bone, which involves reshaping by either the deposition of new bone by osteoblasts or the resorption of bone by osteoclasts at a given site. In the normal bone there is a balance in the remodeling process but this can become dysregulated in disease (Bettica et al 2002, Burr 2004). In the early stages of OA there is an increase in both bone formation and resorption at the bone-cartilage interface,

especially in areas of bone adjacent to damaged cartilage (Wieland 2005). Osteoarthritis can cause an increased rate of bone turnover resulting in architectural changes of the subchondral cancellous bone, particularly an increased bone density and volume (Burr 2004). In patients with hip OA, their trabecular bone volume was found to be increased by 20% (Fazzalari and Parkinson 1997). Fractal signature analysis (FSA) has allowed quantification of the changes in cancellous bone organization, it indicated one of the earliest changes detected in patients with knee OA is a thickening of the horizontal trabecular. As the disease progresses this is followed by thickening of the vertical structures also (Lynch et al 1991, Bukland-Wight et al 1996). The accelerated remodeling causes thickening of the subchondral bone and calcified cartilage layer such that fibrous calcified tissue often advances into the non-calcified articular cartilage layer, which is often seen histologically as the duplication of the tidemark (Wieland 2005, Brandt et al 2006). This causes thinning of the hyaline articular cartilage layer as it becomes replaced by bone, this thinner structure is more susceptible to damage and loss and so exacerbates the progressive degradation of the joint (Brandt et al 2006). The increased subchondral bone density, as a result of increased bone turn over, has been found to have a reduced mineral content making it mechanically weaker (Li and Aspden 1997). The complete loss of articular cartilage and the weakening of the subchondral bone can cause the femoral and tibial articular surfaces to become flattened. In advanced stages of OA, the articular surfaces can become further deformed and distorted which leaded to an altered alignment of the joint (Buckland-Wight 2004, Brandt et al 2006). Subchondral sclerosis occurs initially due to an increased thickness of subarticular cancellous bone, which contributes further to the loss of articular cartilage. This can cause distortion of the articular surfaces, and ultimately results in an increased mechanical load upon the joint (Buckland-Wight 2004).

One of the first definite symptoms of OA that can be seen by radiography is the presence of osteophytes (Neogi et al 2012). Osteophytes are bony projections that form at the joint margins, and are associated with joint space narrowing, sclerosis of the subchondral bone and pain (Conaghan et al 2006). Osteophytes are thought to be a consequence of localized mechanical stress that results in a proliferative response, they start as cartilage outgrowths that become ossified (Felson and Neogi 2004). Osteophytes may cause further malalignment of the joint while also playing a role in the pain associated with OA by putting mechanical pressure on innervated neighbouring joint tissues (Wieland 2005).

As the cartilage is not innervated the pain associated with OA must arise from another structure within the joint, the bone is highly innervated and may be one of the sources of osteoarthritic pain. One feature of OA that has been particularly associated with pain is bone marrow lesions (BMLs) (Felson et al 2001). BMLs have been shown to be associated with the fluctuations of pain seen with knee OA, and pain resolution occurred more frequently if the BMLs became smaller (Zhang et al 2011). Furthermore, BMLs have been shown to be associated with the progression of disease. MRI performed on knees with OA over a 30 month period indicated that enlarging BMLs were strongly associated with an increased loss of articular cartilage (Hunter et al 2006). A second study found the development of BMLs in the knees of middle-aged adults over two years was associated with progressive cartilage pathology, while subjects that displayed resolution of BMLs had reduced progression of cartilage pathology (Davies-Tuck et al 2010). This suggests that BMLs may exacerbate the degradation of cartilage. Furthermore, MRI performed on knees prior to joint replacement surgery indicated that in sclerotic BMLs there is an increased bone volume and trabecular thickness, while the mineral density of lesions was reduced (Hunter et al 2009). This suggests BMLs present a further mechanically compromised region of OA knees that could be susceptible to further damage.

Overall, the altered properties of the bone observed in OA suggest this tissue may now be less capable of absorbing force transmitted through the joint. The mechanically weakened subchondral plate and trabecular bone result in more pressure and subsequent deformation of the articular surfaces. As the disease advances this can result in sub-articular osteoporosis and reduced bone mineral density.

1.3.2.4 The ligaments, meniscus and muscle

The knee joint is a mechanical structure that relies on multiple tissues to maintain its structural and functional integrity. Tissues crucial for knee structure are the ligaments, surrounding periarticular muscle, tendons, and meniscus. The disability and pain associated with OA can be associated with problems in these tissues (Dieppe 2011). It remains questionable if abnormalities in these tissue structures cause the progression of OA or arise as a consequence of the disease.

The medial and lateral collateral ligaments stabilize a joint against valgus and varus stress, therefore it has been suggested that ligament damage and the resultant

instability of the joint could lead to the progression of OA (Brandt 2006). Indeed, Sharma et al found that varus knee malalignment which resulted in an increased medial load in the knee, which subsequently increased the risk of medial OA progression, while valgus knee malalignment resulted in an increased lateral load and increased risk of lateral OA progression (Sharma et al 2001). Similarly, it has been shown that patients with hypermobility syndrome caused by ligamentous laxity are more likely of developing OA (Bird et al 1978). Additionally, MRI studies have shown that anterior cruciate ligament (ACL) rupture was found in 23% of OA patients in a cohort study while this was only present in 3% of patients without knee OA (Hill et al 2005).

The meniscus provides cushioning support between the femur and tibia, and abnormalities in this structure are often found in knee OA (Conaghan et al 2006). Gale et al found with MRI that increasing meniscal sublaxation correlated with the severity of joint space narrowing in patients with symptomatic knee OA (Gale et al 1999). Meniscal damage and extrusion is a further factor that can contribute to knee malalignment, and potentially lead to the development of OA (Hunter et al 2005). Furthermore, the ligaments and meniscus tissues contain type I collagen so also may be in danger of cleavage by enzymes released during cartilage degradation.

The quadriceps muscle provides anteroposterior stability to the knee, weakness of this muscle is common in patients with knee OA, and this could be both an initiating risk factor or a result of the disease. If the quadriceps muscle is weak it may increase the impact of weight loading on the knee joint, which could cause injury and the development of OA. However, the weakening of the quadriceps muscle could be a consequence of the pain that arises when loading an injured OA joint, which leads to patients minimizing the load they place on this joint and could lead to atrophy of the muscle due to lack of use (Brandt et al 2006).

Malalignment of the joint can be caused by ligament damage, meniscal degeneration or position, and muscle weakness, in addition to bone damage and the development of osteophytes. Previously, these factors were thought to be the result of joint damage but are now also considered a potential cause of OA (Dieppe and Lohmander 2005).

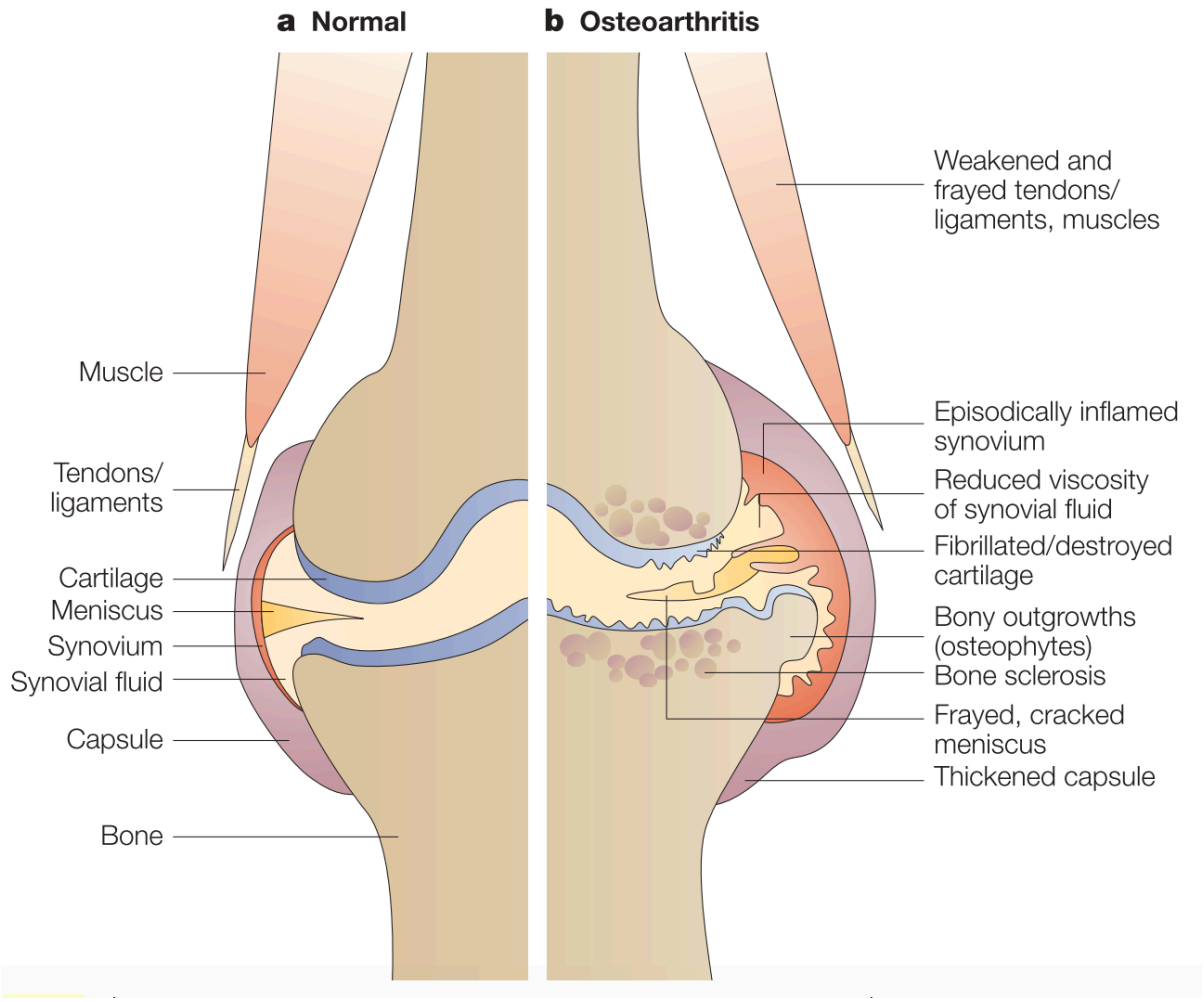


Figure 1.10. The articular structures involved in osteoarthritis of the knee. The image on the left (a) shows the healthy subchondral joint, with a normal smooth layer of cartilage covering the end of each bone, a normal synovium and undamaged bone, meniscus, muscle and ligaments. The image on the right in (b) shows the abnormalities that can occur in the synovial joint during the progression of knee osteoarthritis. The cartilage becomes degraded and fragmented and the synovium becomes enlarged and inflamed. There is also scelorsis of the subchondral bone and the formation of osteophytes at the joint margin. The ligaments, tendons and meniscus can become damaged or ruptured, while the surrounding muscle can become weakened. Figure taken from Wieland et al 2005.

1.3.3 The inflammatory component of Osteoarthritis

OA has been classified as a non-inflammatory arthritis as synovial fluid from OA patients has a leukocyte count below the threshold that defines an inflammatory condition (Dougados 1996). However, the clinical presentation of joint swelling seen in OA is thought to arise from inflammation and thickening of the synovium, and an inflamed synovium is linked with the progression of cartilage damage (Sellam and Berenbaum 2010, Pozgan et al 2010). This indicates that inflammation of the synovial membrane

impacts disease pathogenesis (Sellam and Berenbaum 2010). The release of proinflammatory cytokines also occurs in the cartilage and subchondral bone during the progression of OA, indicating inflammation plays a role across the whole joint. This is shown in Figure 1.11.

The synovial membrane of OA patients displays an infiltration of inflammatory cells composed mainly of activated T-cells, B-cells and macrophages (Sellam and Berenbaum 2010). The inflammatory component of OA pathophysiology is thought to play a large role in early development of the disease as synovial tissue from patients with early OA contain more CD4+ lymphocytes and CD68+ macrophages than patients with advanced OA (Benito et al 2005). This inflammatory response within the synovial membrane can cause an overproduction of cytokines and growth factors. The proinflammatory cytokines produced can then diffuse through the synovial fluid and influence the metabolic state of chondryocytes, contributing to and amplifying the damage of the cartilage matrix (Hedborn and Hausleman 2002, Sellem and Berenbaum 2010).

The proinflammatory cytokines that appear to be the most directly involved in the pathology of OA are IL-1β and TNFα (Westacott and Sharif 1996). The levels of IL-1β and TNFα are elevated in synovial fluid, the synovial membrane, subchondral bone and cartilage in OA patients (Kapoor et al 2011). Chondrocytes, mononuclear cells and osteoblasts can produce these proinflammatory cytokines, but they appear to be produced mainly in the synovium by CD14+ synovial macrophages (Bondeson et al 2006). IL-1 activates cells through binding to the cell surface IL-1 receptor type I (IL-1R1), the expression of which is increased in human OA chondrocytes and synovial fibroblasts (Sadouk et al 1995). The TNF Receptor 1 (TNFR1)/p55 receptor appears to be the dominant cell surface receptor that mediates TNFα signaling, and human OA synovial fibroblasts express higher levels of this receptor than non-OA synovial fibroblasts (Alaaeddine et al 1997). The high expression of these receptors on synovial fibroblasts means IL-1β and TNFα can further stimulate their own production in an autocrine fashion (Sellam and Berenbaum 2010).

The increased level of IL-1β and TNFα in osteoarthritic joint tissues further accelerates cartilage destruction through stimulating the expression of MMPs and ADAMTS in chondrocytes (Kapoor et al 2011, Troeberg and Nagase 2012). The stimulation of human articular chondrocytes with IL-1 results in an increase in the expression of several MMPs (Borden et al 1996). Additionally, the blockade of IL-1β and TNFα in

human OA articular culture resulted in a down-regulation of MMP-1, MMP-3 and MMP-13 expression (Kobayashi et al 2005b). Once IL-1β and TNFα have bound their specific cell surface receptors, their proinflammatory actions are mediated through the activation of MAPK intracellular signaling pathways, and the nuclear factor κB (NFκB) signaling pathway which subsequently mediates the expression of several inflammatory genes (Kapoor et al 2011). This causes an upregulated expression of the genes encoding inducible nitrous oxide synthase (iNOS) and cyclooxygenase-2 (Cox-2), these are enzymes critical for the synthesis of nitrous oxide (NO) and Prostaglandin E2 (PGE2) respectively (Hedborn and Hausleman 2002). Increased levels of NO and PGE2 further enhance the activation and production of MMPs (Kapoor et al 2011). Furthermore, it has been shown in human OA synovium that upregulation of AMAMTS4 was dependent on IL-1β and TNFα (Bondeson et al 2006). The increased secretion of the proinflammatory cytokines IL-1β and TNFα results in the cleavage of collagen and aggrecan in the cartilage ECM.

In addition to upregulating the enzymes responsible for cartilage destruction, IL-1β and TNFα play a role in preventing the synthesis of the cartilage ECM components. IL-1 has been shown to suppress the synthesis of type II collagen in cultured human chondrocytes, and TNFα has been found to inhibit the synthesis of proteoglycan in human cartilage explants (Goldring et al 1994, Saklatvala 1986). During OA inflammatory cytokines are secreted in the joint, this amplifies the degradation of articular cartilage by increasing the expression of destructive enzymes and preventing the synthesis of ECM components.

The increased levels IL-1β and TNFα also promote the production of other cytokines and chemokines through stimulating chondrocytes and synovial cells, amplifying the inflammatory response (Sellam and Berenbaum 2010). Interleukin-17 (IL-17) is a proinflammatory cytokine that can act alone or in concert with TNFα to induce the release of chemokines from chondrocytes and synovial fibroblasts, exacerbating inflammation in the joint (Honorati et al 2006). IL-1β and TNFα can induce de novo synthesis of Interleukin-6 (IL6) in human articular chondrocytes (Guerne et al 1990). IL6 expression has been shown to upragulate the expression of MMP-1 and MMP-13, and consequently promotes collagen release in human and bovine cartilage explants (Rowan et al 2001). IL6 is a particularly important cytokine in bone physiology as it can trigger osteoclast activity and bone resportion, suggesting it may be involved in the altered metabolism of subchondral bone observed in OA (Kwan et al 2004). Increased

levels of further inflammatory mediators such as Interleukin-8 (IL8), Interleukin-10 (IL-10), granulocyte-macrophage stimulating factor (GM-CSF), and Reactive oxygen species (ROS) are also found in OA joints (Kapoor et al 2011, Sellam and Barenbaum 2010).

As the role of the synovium is to provide cartilage with nutrients and oxygen this tissue needs to be highly vascularized. During the progression of OA, angiogenesis occurs in the inflamed areas of the synovium adjacent to cartilage damage. Indeed synovial tissue from patients with OA show increased levels of vascular endothelial growth factor (VEGF) and blood vessel formation (Sellam and Berenbaum 2010, Shibakawa et al 2003). The angiogenic growth factor VEGF is generated by the inflamed synovium, VEGF immunoreactivity in synovial tissue from OA patients was localized with macrophages, and increased with histological inflammation and angiogenesis (Haywood et al 2003). IL-1β, TNFα and IL-17 have been shown to increase the secretion of VEGF from synovial fibroblasts indicating that the inflammatory response in early OA contributes to the development of angiogenesis (Honorati et al 2006). The development of new blood vessels allows additional transport of inflammatory cells to sites of damage in the joint. The processes of inflammation and angiogenesis are closely integrated, with inflammation producing growth factors that stimulate angiogenesis and angiogenesis facilitating inflammation to produce a viscous positive feedback loop accelerating the progression of OA (Bonnet and Walsh 2005).

The initial inflammatory response that occurs in the early stages of OA can trigger events that exacerbate the symptoms of the disease. The proinflammatory cytokines secreted within the joint consequently cause further degradation of the cartilage ECM, angiogenesis within the joint, and alterations in subchondral bone metabolism.

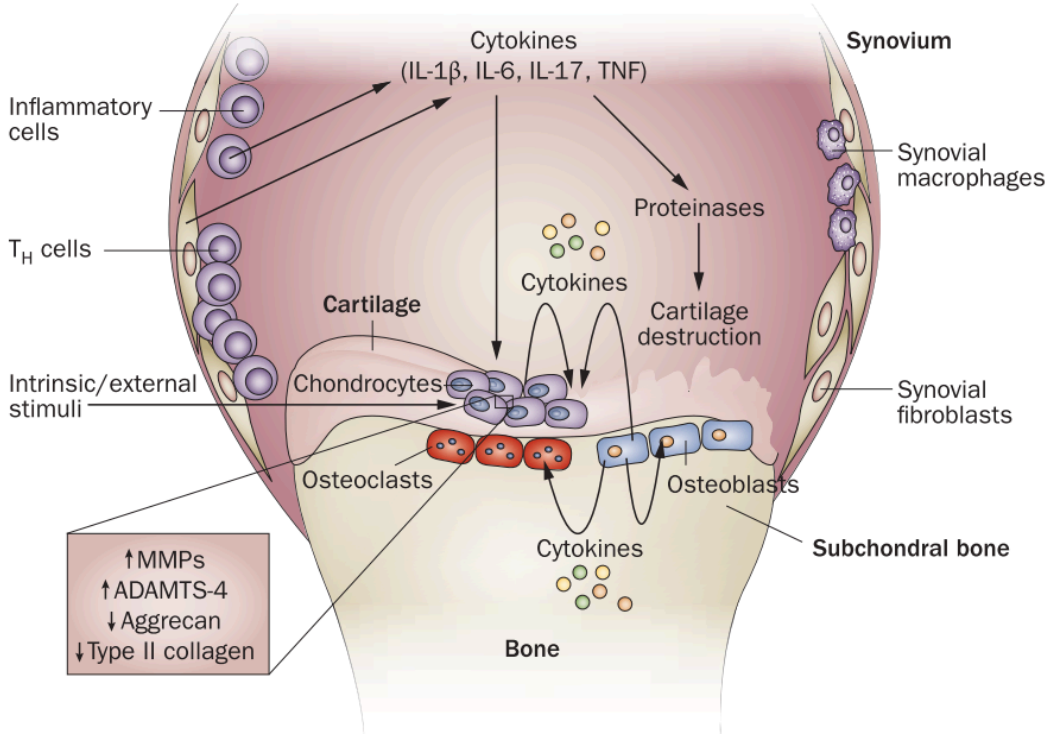


Figure 1.11 The involvement of inflammation during Osteoarthritis of the joint. Inflammatory cells can infiltrate the synovium and release pro-inflammatory cytokines such IL-1β, TNFα, IL-17 and IL-6. The pro-inflammatory cytokines can act on chondrocytes, synovial fibroblasts, and osteoclasts and osteoblasts in the subchondral bone. The cytokine decrease the levels of collage type II and aggrecan in the cartilage through promoting the upregulation of MMP and ADAMTS-4 enzymes and also preventing the synthesis of these macromolecules. (taken from Kapoor et al 2011).

1.3.4 Innervation of the joint

Despite cartilage degradation being the central feature of OA, the articular cartilage contains no neurons meaning any pain associated with OA must be derived in other joint tissues. In contrast to the cartilage, all other joint tissues are densely innervated by small diameter nociceptive neurons (Wieland et al 2005). The clinical finding that OA pain is associated with bone marrow lesions suggests that the subchondral bone may be particularly sensitive to nociceptive stimuli. Retrograde labeling has show that the majority of afferent neurons innervating the subchondral bone were peptidergic neurons expressing CGRP and the receptor for NGF TrkA, both of which are important inflammatory mediators (Aso et al 2014). Interestingly, retrograde nerve tracing has demonstrated a complete absence of the non-peptidergic IB4-binding neurons in the rat knee joint, suggesting that it is unlikely this population of neurons contribute to inflammatory pain in OA (Ivanavicus et al 2004). High threshold nociceptive afferents also terminate in the synovium, this joint tissue contains a vast supply of thinly myelinated and unmyelinated afferent fibres (Mapp 1995, Grubb 2004). The cell bodies

of the joint afferents are located in lumbar DRGs and L3, L4 and L5 levels of the spinal cord (Edoff et al 2000, Ivanavicus et al 2004). The central axons branching from the DRG terminate in two main projection fields in the spinal cord; superficially in LI of the dorsal horn, and in LV-VII of the deep dorsal horn (Craig et al 1988). This allows nociceptive information about forces exerted on articular structures and potential damage to be conveyed to the central nervous system (Grubb 2004).

As the majority of sensory afferents within the joint are peptidergic they can release the neuropeptides CGRP and substance P into the joint upon activation, producing local inflammation (Saito and Koshino 2000, Wieland et al 2005). This paired with the inflammatory mediators released during the development of joint inflammation observed in OA can result in hyperexcitability of joint afferents and leads to peripheral sensitization (Schaible 2004). In cases of persistent inflammation, the hyperexcitability of joint afferents is maintained resulting in an amplification of nociceptive information being transmitted from the joint to the spinal cord. This can lead to central sensitization, where neuroplastic changes in the spinal cord enhance the pain sensation (Wieland et al 2005, Schaible 2004).

1.3.5 Diagnosis

The causes of OA are the microscopic chemically mediated changes and the macroscopic structural changes within the joint. However, clinically it is the symptoms associated with OA that cause individuals to seek medical attention. Joint pain and tenderness, a limitation or stiffness of joint movement, inflammation and effusion are the main features presented with symptomatic OA. Therefore a swollen, tender, or deformed joint tends to be the first clinical sign indicating OA.

Originally OA was confirmed by taking an X-ray of the painful joint, this could be used to identify joint space narrowing caused by cartilage destruction (Dieppe 2011). X-rays can also be used to detect subchondral bone sclerosis and osteophyte formation at the joint margins (McMahon et al 2013, Section 4 Chapter 43). Recently newer imaging techniques, such as ultrasound and MRI, allow visualization of subchondral bone marrow lesions, synovial inflammation, joint effusions and other joint abnormalities that cannot be seen with radiography but contribute to the pathogenesis of OA (Kornaat et al 2006). The detection of joint abnormalities within the joint using MRI is shown in Figure 1.12. Despite the development of more advanced imaging techniques there is a

still a lack of correlation between the intensity of pain and the level of damage observed within the join (Finan et al 2013).

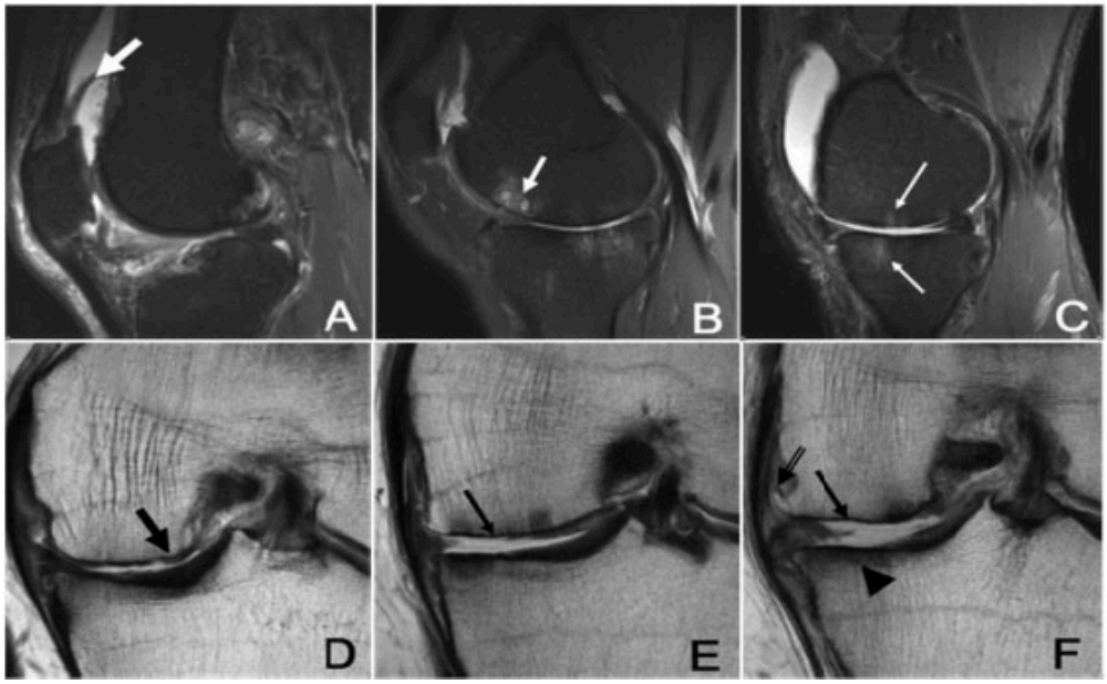


Figure 1.12. Magnetic Resonance Imaging (MRI) of the knee joint and the clinical features observed in osteoarthritis. The arrows indicate the particular features indicative of OA in each panel. Panel (A) shows reactive synovitis, (B) shows the formation of subchondral cysts, (C) shows bone marrow edema, (D) shows partial cartilage wear, and (E) and (F) show complete cartilage loss, subchondral bone sclerosis and osteophyte formation at the joint margin. (Taken from Loser et al 2012).

1.3.6 Epidemiology of Osteoarthritis

Of all of these clinical features associated with OA, pain and disability are the major problems reported by patients. The quality and intensity of pain are variable between patients depending upon the severity and progression of the disease. In the early stages of disease pain is episodic or elicited by movement and weight bearing but is relieved at rest (Schaible et al 2009). However, in advanced cases of OA the pain can be constant, in many cases resting pain is observed through the night (Schaible 2012). Overall, the pain and limited movement within the joint can have a large impact upon an individual's quality of life.

1.3.6.1 Incidence and Prevalence

OA is the most common form of arthritis with a much higher prevalence than rheumatoid arthritis (RA) and one of the most common forms of musculoskeletal disease in the world (Wieland et al 2005). As OA is a disease strongly associated with

aging, longer life expectancy means the incidence is rising and will continue to do so (Pereira et al 2011). The World Health Organization (WHO) state that 10% of men and 18% of women over the age of 60 will have some clinical presentation of OA (Woolf and Pfleger 2003). In the UK, 8.75 million people in the UK seek treatment for OA every year (Conaghan et al 2015). Table 1.3 shows the number of diagnosed prevalent cases in different areas of the world, this indicates the increasing number of cases every five years. It should be noted that a large proportion of patients have radiological evidence of OA but report no pain or symptoms, meaning there may be additional cases of OA that may cause future problems but have not been reported (Srikanth et al 2005).

Table 1.3. Osteoarthritis Epidemiology, the number in millions of diagnosed total prevalent cases. This table is taken from Wieland et al 2005.

Geographical Area	2002	2007	2012
United States	13.2	14.4	15.5
Europe	14.5	15.2	15.8
Japan	6.6	6.9	7.2

1.3.6.2 Economic burden of Osteoarthritis

In a UK wide survey of subjects with self-reported OA, 52% of participants reported that OA had a large impact on their lives (Conaghan et al 2015). Most OA patients have impaired movement to some degree, with knee OA being the major cause of impaired mobility (Woolf and Pfleger 2003). This can compromise an individual's ability to perform their job, indeed 15% of the UK survey participants had taken retirement an average of 7.8 years early (Conaghan et al 2015). The absence from work caused by OA costs the UK economy upwards of £18 billion a year (Conaghan et al 2015). In addition to trouble at work, 25% of patients with OA are not able to perform activities associated with daily life, which can cause a burden on family members and even lead to patients to develop depression (Wieland et al 2005). Furthermore these patients will receive a disability living allowance, further contributing to the economical burden in the UK.

OA, along with the other musculoskeletal diseases, cost the National Health Service (NHS) one tenth of its annual budget (Conaghan et al 2015). OA of the knee and hip contribute the highest burden to immobility and often result in total joint replacement (Cross et al 2014). In Europe, a joint is replaced every 1.5 minute due to OA (Wieland et al 2015). A patient undergoing total knee replacement will cost the NHS £7458, including the 5 years of care they will receive following surgery (Dakin et al 2012).

OA presents an enormous burden upon the individual affected, their family, the NHS and the economy. The costs associated with OA are expected to increase due to the aging population.

1.3.6.3 Risk factors associated with Osteoarthritis

There are a multitude of genetic, environmental, and metabolic triggers that can increase the risk of developing OA. Trauma or physically demanding activities can cause intense mechanical forces to be inflicted on the joint, which may cause injury to joint tissues and initiate the development of OA (Woolf and Pfleger 2003). Systemic risk factors can increase the susceptibility of joints to injury or impair the repair process and so prevent the recovery of damaged joints (Litwick et al 2013). The risk factors identified likely promote the onset of disease and seem to contribute to the development of OA rather than disease progression (Litwick et al 2013). Osteoarthritis usually progresses slowly over a number of years, the associated risk factors may hasten this progression but this is yet to be proven (Litwick et al 2013). The specific risk factors associated with the development of OA are age, obesity, gender, ethnicity, smoking, bone density, muscle function and knee injury. These risk factors are summarized in Table 1.4.

Table 1.4. The risk factors associated with the development of OA.

Risk Factor	Association with Osteoarthritis
Age	Aging is the major risk factor associated with the development of OA, affecting 80% of the population over the age of 75 years old (Arden and Nevitt 2006). This is likely due to progressive cartilage thinning over time, weakened surrounding muscles and the joint generally being less capable of coping with mechanical forces (Zhang and Jordan 2010).
Obesity	Obesity is especially linked to the development of OA in knees and hips mainly due to the increased strain put upon these joints. However obesity can be caused by metabolic dysfunction such as leptin resistance, as leptin regulates bone mass and mineralization a resistance to this hormone may cause an enhanced subchondral bone mass and contribute to the development of OA (Wieland et al 2005, Ducy et al 2000).
Gender	The incidence of knee, hip, and hand OA is higher in women than men and this incidence increases dramatically following the menopause, suggesting hormones may play a role in the development of OA but this is yet to be proven (Srikanth et al 2005).
Ethnicity	The prevalence and joints affected by OA vary among ethnic groups (Litwick et al 2013).

Smoking	There is a negative association between smoking and OA suggesting smoking may play a protective role (Woolf and Pfleger 2003) but it is thought this may to smoking reducing an individuals body mass index (Blagojevic et al 2010).
Bone density	An altered metabolism of the subchondral bone contributes to the pathophysiology of OA. An increased bone mineral density seems to predispose women to developing knee OA (Hart et al 2002). Meanwhile, the reduced bone mineral density that occurs in osteoporosis seems to protect against osteoarthritis (Litwick et al 2013).
Trauma	Physically demanding activities or occupations that may result in joint injury are central risk factors for the development of OA. Indeed, 50% of patients diagnosed with anterior cruciate ligament or meniscal tears developed OA later in life (Lohmander et al 2005).

1.3.7 Current Osteoarthritis treatments

The main reason that patients visit their General Practitioner (GP) for OA is due to the associated pain. A UK report produced in 2012 showed that 71% of patients living with OA are in constant pain, and 1 in 8 of these patients said their pain was unbearable (Smith 2012). Therefore the primary concern for GPs is alleviating this pain, however 2 in 5 patients in the UK said their treatment was ineffective (Smith 2012). There are a number of pharmacological interventions available to provide pain relief. However in advanced OA cases, if these pharmacological interventions prove ineffective and joint damage causes complete immobility the final option is surgical intervention and total joint replacement, which will require further substantial pain management.

1.3.7.1 Pharmacology

Paracetamol

Paracetamol is the first-line oral analgesic recommended for treating the pain associated with knee OA (Wieland et al 2005). Up to 4g a day is though to provide relatively effective pain relief and have limited side effects (Zhang et al 2004). In two placebo controlled trials, one showed paracetamol to be more effective at alleviating pain than placebo, but the other showed that paracetamol was no better at treating pain than placebo (Zhang et al 2004). This suggests that despite its safety and low risk of side effects, it may prove ineffective in many patients.

NSAIDs

For patients who do not respond to paracetamol the next line of treatment to be considered are non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs function by

targeting the cyclooxygenase isoforms Cox-1 and Cox-2, which are key enzymes in the synthesis of prostaglandins. The synthesis of prostaglandins at sites of inflammation is thought to contribute to peripheral sensitisation (See section 1.1.4). Prostaglandins can bind to cell surface receptors on primary afferents to initiate intracellular signaling cascades; the subsequent activation of intracellular PKA mediates phosphorylation of sodium channels in nociceptor terminals (Wieland et al 2005). The phosphorylation of sodium channels lowers their activation threshold and causes increased excitability of peripheral nociceptors. Therefore through preventing the synthesis of prostaglandins, NSAIDs target peripheral sensitization that arises due to the inflammatory component of OA. Topical NSAIDs have been shown to be effective at reducing knee OA pain for a short period of time, but their effectiveness was reduced over time with continual application (Lin et al 2004).

The Cox-1 isoform is constitutively expressed in many cell types, while the expression of the Cox-2 isoform is induced by pro-inflammatory cytokines and growth factors meaning it is often present at sites of inflammation (Wieland et al 2005). Despite the pain sensitizing effects of prostaglandin during inflammation, prostaglandins can have important protective effects. Protective prostaglandins are synthesized by Cox-1 to preserve the integrity of the stomach lining and are also required for normal renal function (Vane et al 1998). Therefore, oral NSAIDs can have gastrointestinal side effects including ulcerations and perforations (Russell et al 2001). However, drugs designed to specifically target the Cox-2 isoform can reduce inflammation without removing the protective prostaglandins from the stomach (Flower 2003). Cox-2 specific inhibitors are as effective as traditional NSAIDs but cause fewer gastrointenstinal complications (Laine et al 2008).

Opioids

If both paracetamol and NSAIDs prove ineffective at relieving OA pain opioid analgesics, such as Tramadol, can be the next alternative. Opioids have been shown to be effective at relieving pain and restoring physical function in OA, but they also come with adverse side effects such as dizziness and drowsiness (Avouac et al 2007). These side effects can be particularly dangerous in the elderly. Due to the adverse side effects and the development of tolerance that can lead to potential abuse, opioids are not considered to be particularly safe and should only be considered for short-term pain relief.

Corticosteroids

For patients suffering with episodic inflammation within the damaged joint intra-articular injections of corticosteroids may provide anti-inflammatory action. Intra-articular corticosteroids have been shown to reduce joint pain and disability while relieving inflammation for up to 1 week, and this is accompanied with very few side effects (Bellamy et al 2006, Hepper et al 2009). However this only provides short-term relief, so should only be used occasionally to supplement paracetamol/NSAIDs.

Hyaluronan

Hyaluronan is synthesized by synoviocytes, fibroblasts and chondrocytes, and plays an important physiological role in the joint aiding lubrications and inhibiting the synthesis of prostaglandins (Brandt et al 2000). In OA the concentration of hyaluronan in the synovial fluid is reduced, which may contribute to the compromised function of the joint (Brandt et al 200). Intra-articular injections of hyaluronic acid are designed to compensate for this deficiency. However when intra-articular hyaluronic acid injections were compared to placebo injections, they were found to only have a small effect in treating knee OA (Lo et al 2003).

Glucosamine and chondroitin sulphate

Both glucosamine and chondroitin sulphate are compenents of the cartilage ECM, they are marketed as nutritional supplements that may have structural-modifying effects in OA (Reginster et al 2003). Although both have been reported to potentially prevent joint space narrowing over time, they are relatively ineffective at relieving the symptoms of OA (Richette and Bardin 2004, Reichenbach et al 2007).

1.3.7.2 Surgical intervention

When pharmacological interventions have failed to provide any pain relief or improvement in function, the final stage treatment for OA is a total joint replacement. During this surgical procedure the ends of the articular structures are removed and replaced with synthetic materials such as metal, plastic or ceramic. This is a relatively cost-effective procedure in terms of the number of quality-adjusted life years that can be gained (Dakin et al 2012, Rasanen et al 2007). Total joint replacement has been shown to dramatically improve joint function and relive joint pain with the greatest improvements seen 3-6 months following surgery (Ethgen et al 2004). Recovery

following the surgery usually takes 6-10 weeks and the prosthetic joints usually remain functional for approximately 10 years.

Despite this surgical intervention being designed primarily to alleviate the chronic pain that can accompany OA, the surgery can leave patients with post-operative pain. Wylde et al found that in patients who had undergone total knee replacement or total hip replacement between 2004-2006, 44% and 27% suffered from some form of post-operative pain respectively (Wylde et al 2011). It has been found that females, younger patients needing total joint replacement, patients with a high BMI, and patients with a higher pre-operative pain score are most likely to develop post-operative pain (Singh et al 2006, Liu et al 2012). Additionally, as OA patients are often elderly or obese this can complicate the surgery, result in inferior improvements, or increase the likelihood of mortality following surgery although the risk is small (Amin et al 2006).

1.3.8 Evidence of central sensitization in Osteoarthritis

The treatment options for OA discussed above all aim at tackling the pain at the peripheral source, however there are features of the pain associated with OA that cannot be explained by a purely peripheral mechanism. Patients often develop referred pain in an area distant to the damaged joint, and even though total joint replacement relieves the pain caused by OA in the majority of patients a subset of patients are left with pain following joint replacement (Wylde et al 2011). Additionally, multiple animals models of OA have highlighted altered central nociceptive processing (Orderberg 2004). This suggests patients may develop central sensitisation as OA progresses.

As discussed previously, the inflammatory mediators released in the joint during OA can cause sensitization of nociceptors innervating articular structures (See section 1.1.4, section 1.3.3, and section 1.3.4). The subsequent hyperexcitability of joint primary afferents can result in an increase in the intensity and number of pain signals being sent to the dorsal horn. The neuroplasticity that exists in the central nervous system may result in second order neurons in the spinal cord becoming hyperexcitable or for an increased activation of supraspinal structures, which may result in an amplified pain response (Pinto et al 2007, Gwilym et al 2009). Together these mechanisms could be attributed to a long-term centrally dysfunctional amplification of the pain response.

The plasticity that exists within the somatosensory system upon the development of chronic pain begins in the dorsal horn (DH). The expression of c-fos within the spinal

cord was found to be higher in a chronic articular inflammatory rat model compared to an acute articular inflammatory model, which suggests spinal cord neurons were abnormally active during the induction of chronic inflammatory pain (Pinto et al 2007). Patients with chronic knee OA have extended areas of referred and radiating pain in response to hypertonic saline compared to control subjects (Bajaj et al 2001). The appearance of referred pain is delayed compared to local muscle pain, indicating the mechanism behind this phenomenon is time-dependent (Graven-Nielsen 2006). The persistent noxious input from the osteoarthritic joint may allow latent synaptic connections to become operational, and afferent terminal connections spread over several segments of the spinal cord to become activated, allowing convergence of input and development and expansion of the receptive field. The release of sensitizing neuropeptides, such as substance P, around second order neurons in the dorsal horn may contribute to the expansion of the receptive field. Indeed animal studies have shown peripheral tissue damage and inflammation to alter the neurochemical state of excitatory amino acids at the spinal level to induce activation of the NMDA receptor (Dickenson et al 1997). Amplification of pain signals within the DH, and the unmasking of new receptive fields due to central sensitisation may be mediating the referred pain that develops in OA and explain why the pain perceived does not always match the peripheral stimulus (Graven-Nielsen 2006).

The increased excitability within the spinal cord may be accompanied by supraspinal changes that contribute to central sensitisation. The use of fMRI has indicated increased activation of brainstem areas upon application of noxious stimulation, but this can only be observed in states of central sensitisation (Lee et al 2008). This technique has also shown increased activity within the PAG when referred pain areas were stimulated in patients with hip OA, indicating brain biomarkers for central sensitisation in patients with chronic OA (Gwilym et al 2009). The brainstem is also the area in which descending systems arise that can modulate the excitability of spinal neurons. A further clinical feature observed in OA patients is secondary hyperalgesia where sites away from the joint become sensitive to noxious and innocuous stimuli. In a rat model of OA, the descending serotonergic faciliatory system was shown to play a role in mediating the responses of spinal neurons to innocuous stimuli in the area of secondary hyperalgesia (Rahman et al 2009). This indicated that adaptive changes in the brainstem and subsequent alterations in the descending modulatory pathways, may mediate the amplified responses of spinal neurons to noxious and innocuous inputs.

Alterations with the central nervous system that may contribute to the development of chronic pain in OA are discussed in greater detail in chapters 5 and 6.

The development of chronic pain as a result of OA may be attributed to both peripheral mechanisms due to the release of inflammatory mediators within the joint, and subsequent centrally mediated mechanisms. Both mechanisms should be considered when deciding on the appropriate analgesics used to target pain in OA patients.

1.4 Thesis Aims and Hypothesis

The specific pathways and mechanisms subserving diffuse noxious inhibitory controls (DNIC) remain unclear, understanding these mechanisms may allow us to better understand the central changes that occur during the development of chronic pain. Many patients with osteoarthritis develop clinical features to their pain that suggest complex changes in the central nervous system are involved, and the analgesics currently available to osteoarthritis patients remain relatively ineffective. Investigating DNIC in a rat model of osteoarthritis may indicate the central changes involved in the development of chronic pain. Through better understanding of the mechanisms involved in the development of chronic pain during the progression of OA, we may be able to target and manipulate these mechanisms to provide better analgesia to OA patients.

The experiments in this thesis aimed to:

- Develop a model for assessing DNIC at the spinal level, using recordings from convergent wide dynamic range neurons in the deep dorsal horn.
- Further unravel the roles of noradrenaline and serotonin in mediating DNIC.
- Investigate potential changes in DNIC in a rat model of osteoarthritis.
- Understand the processes that may be leading to a loss of DNIC during the development of chronic pain in OA.
- Pharmacologically manipulate DNIC in a rat model of osteoarthritis; investigating potential therapeutics for OA patients who suffer from chronic pain due to altered central processing of nociception.

Hypothesis:

- A conditioning noxious pinch will produce WDR neuronal inhibition in response to a multiple stimulus modalities, including mechanical, thermal and electrical.

- The noradrenergic descending pathway acting at α_2-adrenoceptors in the dorsal horn results plays an anti-nociceptive role and enhances the magnitude of neuronal inhibition induced by DNIC, while the release of serotonin from descending pathways in the dorsal horn plays an pro-nociceptive role and inhibits the neuronal inhibition induced by DNIC.

- As alterations in the descending noradrenergic and serotonergic systems have previously been demonstrated in the MIA model, the neuronal inhibition induced by DNIC will be lost.

- There is a neuropathic component to the 2mg MIA model of OA.

2. Materials and Methods

2.1 In vivo electrophysiology

2.1.1 Animals

All animal experiments were approved by the United Kingdom Home Office and were carried out in accordance with guidelines set by personal and project licenses and thus complied with the UK Animals (Scientific Procedures) Act 1986. Every care was taken to reduce the suffering of animals and the number of animals used in accordance with the guidelines provided by the International Association for the Study of Pain (IASP) for the care and use of laboratory animals.

Male Sprague Dawley rats were housed in the UCL Central Biological Unit. Food and water were provided ab libitum in cages that were under a 12 hour light/dark cycle. The weight of rats used for electrophysiology experiments were between 220-290g.

2.1.2 Preparation

The protocol used has been previously described (Urch and Dickenson 2003). Rats were anesthetised in an induction box with 4% isofluorane carried in 66% nitrous oxide and 33% oxygen. Once the rats were fully unconscious and a-reflexive, they were removed from the box and placed on a nose cone to continue anaesthetic delivery. Isofluorane anesthetic was dropped to 3% while a tracheotomy was performed. The trachea was exposed through dissection and a cannula inserted, which was secured with silk thread. The anesthetic was then dropped to 2.5% and was delivered via this cannula for the rest of the experiment.

The rats were secured into a stereotaxic frame including ear bars and a laminectomy was performed. Within the stereotaxic frame the rat was placed onto a homeothermic heat mat and a rectal temperature probe was inserted in order to maintain the animals core temperature at 37°C.

For the laminectomy an incision was made into the skin along the length of the back. The bottom of the ribs were located and incisions were made either side of the vertebral column such that the spinal cord could be clamped securely. Rangeurs were used to remove muscle and vertebrae and expose L4-L5 segments of the spinal cord. The dura was removed. A well of muscle bone and connective tissue were left surrounding the

spinal cord such that 50μL drug aliquots could be applied spinally. Below the laminectomy, two more incisions were made either side of the spinal column to place the second clamp towards the bottom of the cord. Crucially, the cord was straight and held tightly and securely such that no movement was evoked by respiration. Following the laminectomy the isofluorane was lowered to 1.5% and maintained at this level for the duration of the experiment.

2.1.3 Single unit recording

Spinal neuronal recordings were made from wide dynamic range (WDR) neurons located in the deep dorsal horn. A headstage containing a parylene-coated tungsten electrode (125μM diameter) was lowered into the spinal cord to a depth of approximately 500μM-1000μM in correspondence with the expected depth of lamina V using a micromanipulator. Within the headstage were two further connections used to ground the system. Firstly, a grounding lead was attached to the stereotaxic frame and collected background electrical activity. The second grounding lead was fixed to the animal to collate electrical information not connected to spinal cord activity such as heart beat. This information was fed into a NeuroLog AC recording system (NeuroLog system, Digitimer, UK) set at the A-B setting such that the input from the grounding cables was subtracted from that of the electrode. The incoming signal was band-passed filtered with the low/high frequency cut off set at 1.5-2KHz, as this frequency band allowed for clear differentiation of action potentials.

To identify an individual WDR neuron the electrode was slowly lowered through the dorsal horn while the receptive field was stimulated through light tapping. Once an area of evoked potential was discovered the electrode was adjusting slightly to ensure a single unit had been isolated. A single unit is represented on the osciliscope as one consistent amplitude of action potential firing with no interference from adjacent cells. Once a single cell was identified, its responses to different stimuli were quantified. Neurons were confirmed as WDR if they responded to both innocuous (8g) and noxious (60g) mechanical stimulation, thermal stimulation of 48°C and exhibited wind-up. Data was captured using Spike 2 software coupled to a CED 1401 interface (Figure 2.1). Action potentials fired were displayed in a histogram format.

Responses were recorded to natural mechanical and thermal stimuli. For mechanical stimulation von Frey hairs (Touch-Test, North Coast Medical, USA) of increasing bending force (8g, 26g and 60g) were applied to the receptive field (the hind paw). For

thermal stimulation a water jet (42°C, 45°C, 48°C and 50°C) was applied to the receptive field. Natural stimuli were applied for a period of 5 seconds per stimulus and there was a 60 second separation between the application of each stimulus. All WDR neurons met the inclusion criteria of <10% variation in action potential firing for all evoked neuronal responses An example trace of one WDR neuron firing in response to natural stimulation is shown in Figure 2.2.

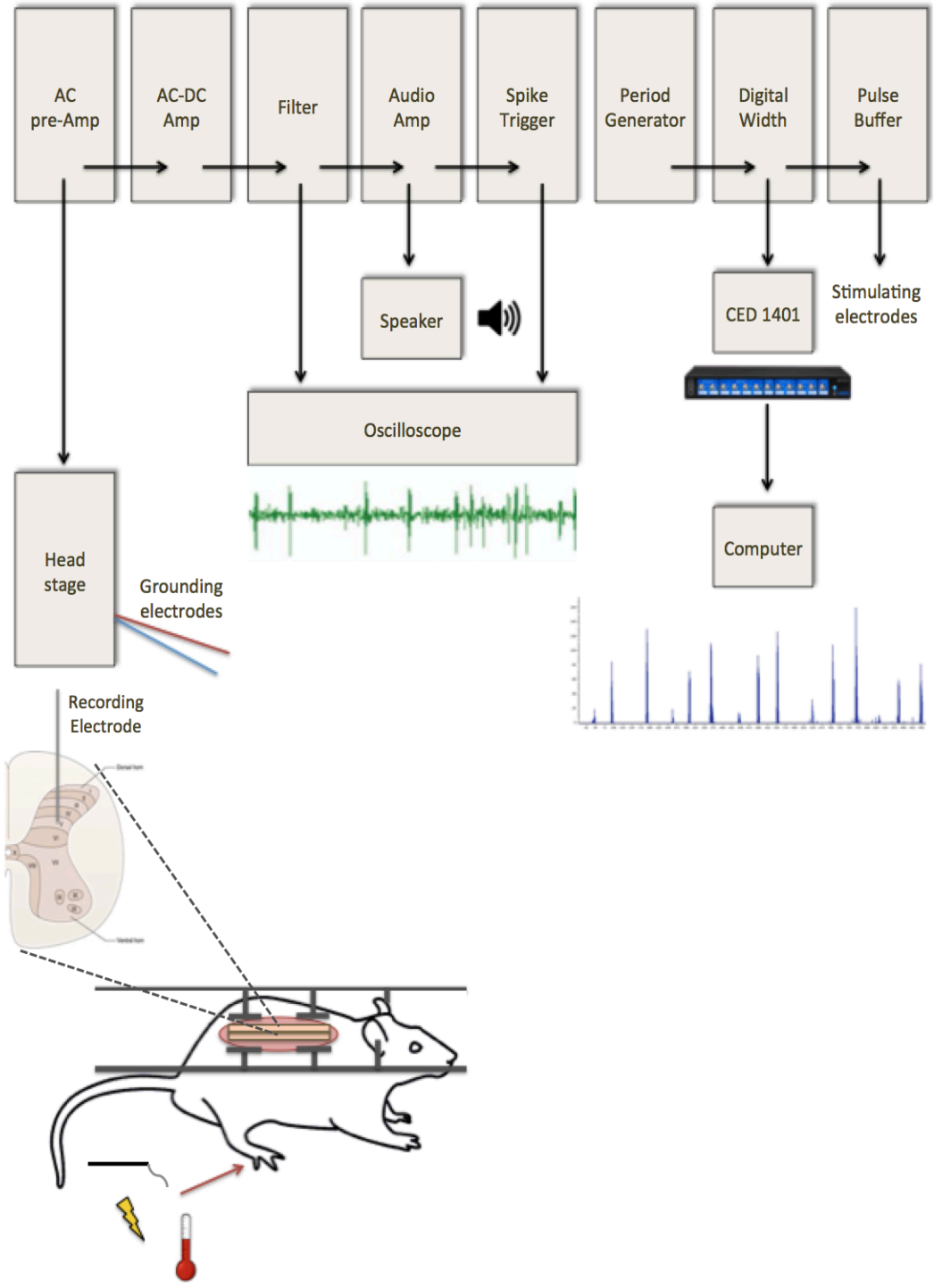


Figure 2.1 Schematic of electrophysiological and Neurolog recording system. A laminectomy was performed to expose L4-L5 segments of the spinal cord. The receptive field of

the hind paw is stimulated with natural or electrical stimulation. Recordings were made from WDR neurons in the deep dorsal horn of the spinal cord with a tungsten electrode. The input from the recording electrode enters the recording system via a headstage. Background electrical activity is subtracted and the signal is amplified, filtered and fed into a speaker and oscilloscope so the cell can be heard and visualized to ensure a single unit has been isolated. Action potentials with amplitude above a determined threshold were recorded and output to the CED 1401 interface, which were quantified via the computer. Electrical stimulations were delivered to the receptive field via two stimulating electrodes. Electrical inputs were captured and converted into a post-stimulus time histogram to provide information on the fibre types.

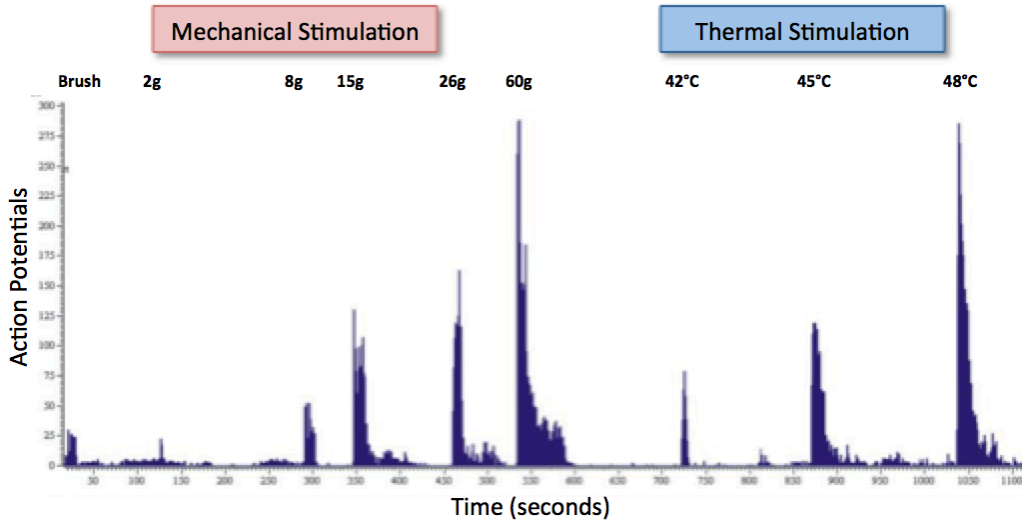


Figure 2.2 Responses of a WDR neuron to natural stimulation. A representative histogram trace from a single WDR neuron to show the coded range of responses to a range of natural stimulation.

Single electrical pulses were delivered in increasing mA increments to determine the minimum stimulating threshold for C fibres. A train of 16 pulses were delivered to the receptive field (2ms wide pulse at 0.5Hz) at 3 times the C fibre threshold. A post-stimulus time histogram (PSTH) was constructed to quantify and separate responses evoked by peripheral afferent fibres based on their latency (Figure 2.3). This was 0-20ms for Aβ fibres, 20-90ms for Aδ fibres, and 90-300ms for C fibres. Any activity that occured after C fibre latency (anything from 300ms to 800ms) was calculated as the post discharge (PD).

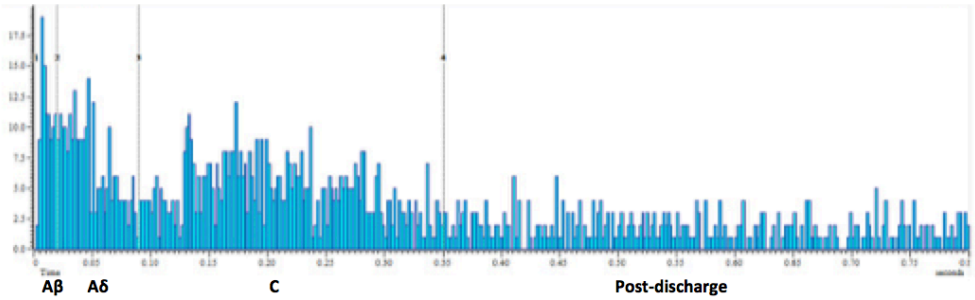


Figure 2.3 Response of a WDR neuron to electrical stimulation. An example post-stimulus time histogram (PSTH) from a single WDR neuron responding to electrical stimulation. The PSTH separated fibre types based on the latency of the evoked potential. Aβ fibres are classed as having a latency of 0-20ms, Aδ fibres between 20-90ms, and C fibres between 90-300ms. Evoked activity measured post 300ms is measured as post-discharge.

A predicted no wind-up response is calculated by multiplying the number of action potentials fired by the first electrical stimulation by 16. This hypothetical value is termed Input and is what we would expect if a cell had no change in the evoked response with subsequent stimulations over the 16 pulse period (Figure 2.4). Wind-up was calculated by taking the input value from the actual accumulative count of action potentials fired from the 16 stimulations:

Wind-up = total number of action potentials fired after 16 stimuli – Input

A rest period of 10 minutes was allowed between each round of electrical stimulation to prevent sensitisation of the receptive field and to ensure that the neuronal responses had returned to baseline.

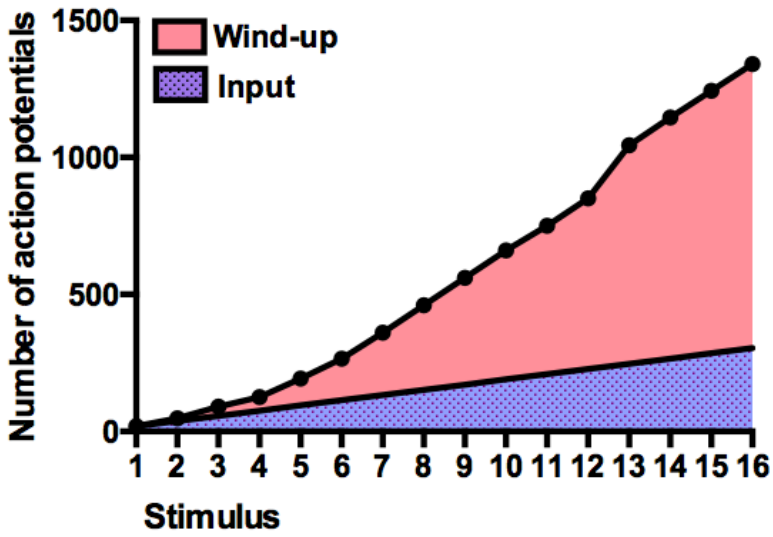


Figure 2.4 An example of wind-up in a single WDR neuron. The number of action potentials fired by a single WDR neuron in response to 16 consecutive electrical pulses delivered at 3 times

the C fibre threshold were quantified. The theoretical input (purple) is what we would expect if a neuron exhibited no change in evoked response with subsequent electrical stimulations. The actual number of action potentials fired (pink) shows that with each subsequent electrical stimulation an increasing number of action potentials are fired.

To validate that setting the threshold was a sufficient method for ensuring recordings were taken from a single unit, an experiment was performed on the Spike2 software usually used for brain recordings. This programme allowed recordings of multiple neurons and separated them based on their waveform. It was considered that during intense noxious stimulation other neurons might begin firing such as nociceptive specific (NS) and this might count towards the action potential count histogram. This programme characterizes specific waveforms and expresses them as separate colours. When the isolated WDR neuron was mechanically stimulated with von Frey hairs of increasing intensity, only one type of neuron was detected on the Spike2 software (Figure 2.5). This suggested that monitoring the threshold carefully throughout the experiment was a sufficient method for ensuring action potentials fired were resulting from a single unit.

At the end of each study, the isofluorane was set to 5% to overanaesthetise the rat and a cervical dislocation was performed to confirm death.

2.1.4 Pharmacology

Detailed protocols for pharmacology experiments including doses and time points are given in individual chapter methods.

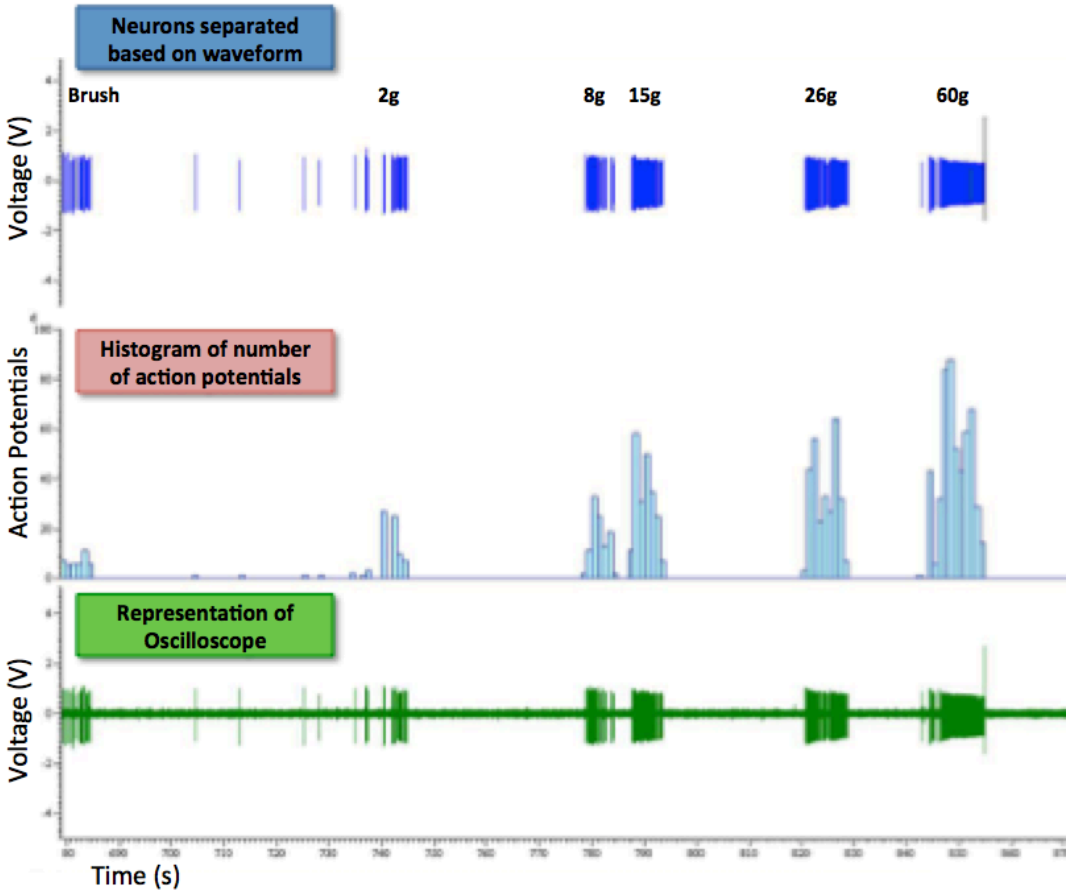


Figure 2.5 Recordings taken from a WDR neuron using Spike2. The top panel represents neuronal populations based on their waveform, it expresses each neuronal population as a different colour. The middle panel shows the number of action potentials fired over a determined threshold and expressed as a histogram. The bottom panel is a representation of the oscilloscope. Each time the receptive field is stimulated, the oscilloscope displays a change in voltage due to neuronal action potentials. As the recordings are extracellular, the change in voltage displayed is relative to that outside the neuron.

2.2 DNIC experiment design

2.2.1 Mechanical stimulation

Firstly, the pre-conditioned mechanically evoked neuronal firing rates were quantified in response to 8g, 26g and 60g von Frey filament stimulation applied to the hind paw. This was repeated 3 times to obtain a stable average pre-conditioned response value. For the DNIC response, the von Frey filaments were applied to the receptive field in the presence of a concomitant noxious ear pinch (15.75 x 2.3 mm Bulldog Serrefine, Interfocis, Linton, UK). The DNIC response was characterized as the number of action potentials fired when the receptive field was stimulated alone and compared to the number of action potentials fired in the presence of a concurrent noxious conditioning

pinch. This counted as one DNIC trial, which was repeated such that the baseline responses were calculated as the mean of the two trials. In MIA animals, DNIC was also induced with a noxious knee pinch, where a clamp was placed around the knee and the two prongs pushed together to a set distance of 0.75cm.

2.2.2 Thermal stimulation

The number of action potentials fired when a water jet (42°C, 45°C, 48°C and 50°C) was applied to the hind paw was quantified. This was repeated twice to obtain an average stable pre-conditioned response. DNIC was then induced by stimulating the hind paw with a water jet of a specific temperature in the presence of a concomitant noxious ear pinch. This DNIC trial was repeated and the mean values from the two trials were used as pre-conditioned and DNIC responses.

2.2.3 Electrical stimulation

A train of 16 pulses were delivered to the receptive field (2ms wide pulse at 0.5Hz) at 3 times the C fibre threshold. This was repeated 3 times with a 10-minute break between each train of 16-pulse stimulations. The same 16 pulses of electrical stimulation were then applied to the hind paw in the presence of a concomitant noxious ear pinch. This was counted as one DNIC trial and repeated to obtain the average pre-conditioned and DNIC responses.

2.3 Experimental Pain Models

2.3.1 Spinal Nerve Ligation

The L5/L6 spinal nerve ligation (SNL) model has been previously described by (Kim and Chung 1992). Male Sprague-Dawley rats weighing approximately 120g were anesthetised in 3% isofluorane delivered in 66% nitrous oxide and 33% oxygen for induction and the isofluorane was maintained at 2% for the remainder of the surgery. Under aseptic conditions, a small left hand incision was made at approximately L4-S2. The left tail muscle and fat were removed to expose the L5 and L6 spinal nerves. The L5 and L6 spinal nerves were then isolated with a fine glass hook and then tightly tied with non-absorbable 6-0 braided silk thread. The surrounding skin and muscle were closed with absorbable 3-0 sutures.

For all SNL animals used in Chapter 4, the surgery was performed by Dr Leonor

Gonçalves. For this study, half of the WDR neuronal recordings were contributed by Dr Kirsty Bannister as part of a collaborative project.

2.3.2 Monoiodoacetate induced arthritis

The Monoiodoacetate (MIA) model of osteoarthritis was originally described by Kalbhen and colleagues (Kalbhen et al 1987). Male Sprague-Dawley rats weighing 190-210g (Early phase) or 120-140g (Late phase) were anaesthetized with 3.5% isofluorane delivered in 66% nitrous oxide and 33% oxygen until the animals were fully unconscious and a-reflexive. Animals were then placed on a heated mat, and the isofluorane anaesthetic was delivered at 2% via a nose cone for the remainder of the surgery. The ventral surface of the hind limb was clipped of hair around the knee area and cleaned with clorhexidine. The joint was flexed in the left knee and arthritis was induced by an intrarticular injection of 2mg MIA in 25μL of 0.9% saline. The MIA was administered with a 27g needle through the infrapatellar ligament, the needle was held in place for 30 seconds and withdrawn slowly to avoid any leakage of the MIA. For Sham animals, 25μL of 0.9% saline was injected. Rats were placed in an incubator to recover, and subsequently in clean cages and monitored for the next 72 hours. The day of injection was considered day 0. Early phase and Early phase Sham animals were used for electrophysiology 2-6 days post injection, while Late phase and Late phase Sham animals were used 14-20 days post injection.

2.4 Behavioural Testing

For all behavioural testing the experimenter was blinded as to which were MIA injected animals and saline injected sham controls.

2.4.1 Acclimatization

Before any behavioural testing animals were allowed to acclimatize to the equipment used. This was carried out on two consecutive days before baseline responses were recorded. Equipment used for paw withdrawal threshold testing includes a rack of Perspex boxes open at the top and bottom and placed on a wire mesh rack. Rats were placed in individual boxes and given 20 minutes to acclimatize to their surroundings. For weight bearing an incapacitance tester (Linton Instrumentation, Norfolk, UK) was used as described by (Bove et al 2003) where the animals are placed in a clear angled Perspex chamber. In the chamber the animals placed each hind paw on separate force

platforms, with their tail placed out of hole in the back, and their front hind paws on the Perspex surface as depicted in Figure 2.5. During the acclimatization days the rats were trained to stand with equal weight on each platform. During acclimatization and behavioural testing the room was booked to prevent interruption so that a quiet and undisrupted environment was provided.

2.4.2 Mechanical Hypersensitivity

Rats were placed in individual Perspex boxes within the rack and allowed to acclimatize for 10 minutes before testing. Mechanical hypersensitivity was tested at the site of the hind paw in order to assess the presence of secondary hyperalgesia as a result of the MIA injection.

For the first method used to test mechanical hypersensitivity, von Frey hairs of increasing intensity were applied to the plantar surface of the hind paw (1g, 2g, 4g, 6g, 8g, 10g, 15g and 26g). If the animal removed its paw from the von Frey hair stimulation all 5 times the testing stopped and this was noted as the animals paw withdrawal threshold (PWT). From this point von Frey hairs of higher bending force were not used on this animal. This was performed on both ipsilateral and contralateral hind paws with ipsilateral testing performed first on all rats followed by testing on the contralateral paw.

The second method used to test mechanical hypersensitivity, the up-down method, has been previously described by (Chaplan et al 1994). Von Frey filaments were applied to the plantar hind paw for 5 seconds and any withdrawal from this, including flinching or shaking, counted as a positive response. The von Frey hairs used in this test were 1.4g, 2g, 4g, 6g, 8g, 10g and 15g. To begin with the 6g stimulation was tested and subsequently the weight of the next von Frey filament used would be greater or less depending on a negative or positive response respectively. Following a positive response, 4 more von Frey filaments were tested, either increasing or decreasing in weight depending on responses. 50% paw withdrawal thresholds (PWT) were then calculated with the following formula: PWT = (10^(x + kδ)/10,000), where x represents the log of the last von Frey tested, δ represents the mean difference between the von Frey filaments in log units (0.17) and k, a value dependent on the series of responses (Dixon et al 1965). This was performed on both ipsilateral and contralateral hind paws. Ipsilateral testing occurred first, with a full test carried out on one animal to determine PWT, this was then continued on the rest of the rats before returning to the first rat to

repeat the tests on the contralateral hind paw.

Baseline PWT was carried out twice on two consecutive days and the average taken. For early phase animals tests were then carried out on day 2 and day 4 post injection. For late phase animals tests were carried out on day 2, day 4, day 7 and day 14 post injection.

2.4.3 Weight bearing assessment

Animals were placed in the angled Perspex chamber on the incapacitance tester and were given 5 minutes to acclimatize. Once animals were stood in the correct position with one hind paw in contact with each force platform, the force exerted by each hind paw was measured over a 5 second period. This was repeated 3 times with each animal. From these 3 recordings, the average weight placed on the ipsilateral limb was quantified and expressed as a percentage of both hind paws. Weight bearing assessment was only performed in animals that remained calm; if an animal seemed stressed in the equipment, assessed through vocalization, it was returned to the cage and tested again after recovery.

Baseline weight bearing was obtained from the average of two trials performed on consecutive days. For early phase animals further tests were carried out on day 2 and day 4 post injection. For late phase animals further tests were carried out on day 2, day 4, day 7 and day 14 post injection.

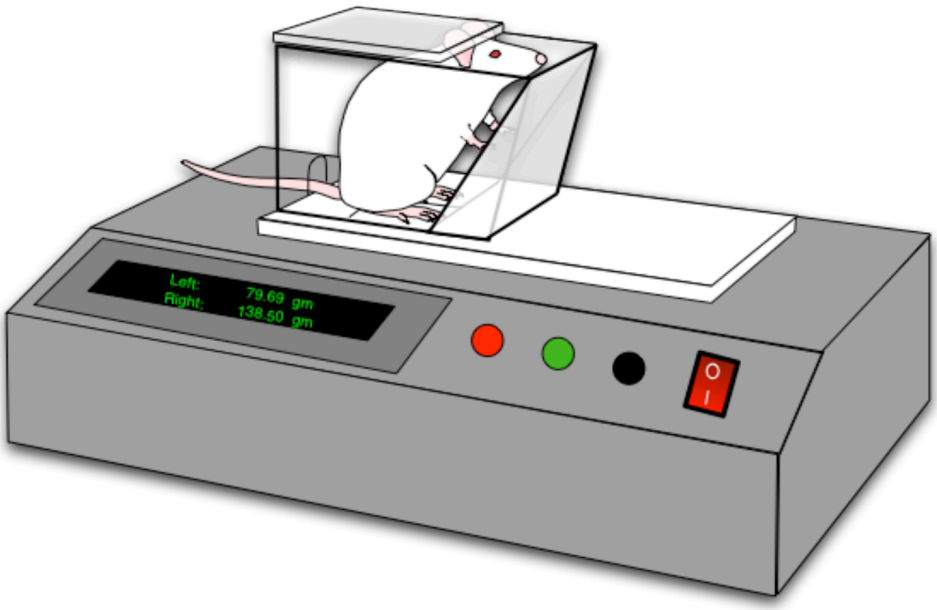


Figure 2.6 Weight bearing assessment using a incapacitance tester. The animal was placed in a Perspex box with the front paws leaning on the angled surface while each hind paw was

placed on a separate panel on the incapacitance tester. Once the rat was in the correct position, the panels measured how much weight the rat was placing on each hind paw in grams. Image gifted by Dr Louisa Townson.

2.5 Knee Histology

2.5.1 Dissection, fixation and decalcification

At the end of electrophysiology experiments, the isofluorane was increased to 5% to over-anaesthetise the animal and a cervical dislocation confirmed death. Following this the knee joint was roughly dissected with the tibia and femur and any excess muscle or tissue surrounding the joint was removed. It was not necessary to perfuse the animal before hand as the cartilage contains no blood vessels so perfusion would not have help preserve this particular tissue. Following dissection the entire knee joint was placed into 4% paraformaldehyde (PFA) for 24-48 hours for fixation.

Following fixation the knees were rinsed in water for 30 minutes on a shaker. The knee joints were then dehydrated with steps of increasing concentrations of ethanol. Firstly, in 30% ethanol for 30 minutes, then in 50% ethanol for 30 mins and finally placed in 70% ethanol. For all of these steps the knees were placed on a shaker. At this point the knees were stored until they were ready to be processed.

Before decalcification the knees were rehydrated; they were placed in 50% ethanol for 30 minutes, followed by 30% ethanol for 30 minutes, and finally were placed into water. The knees were then placed in Immunocal® (Quarttet, Berlin, Germany) for decalcification for approximately one week. To check the progress of decalcification the joints were checked using a microCT (SkyScan 1172) on day 3 and day 7 (Figure 2.6). This was used to confirm the joint was completely decalcified as any calcium remains would cause the knee to be too brittle to section.

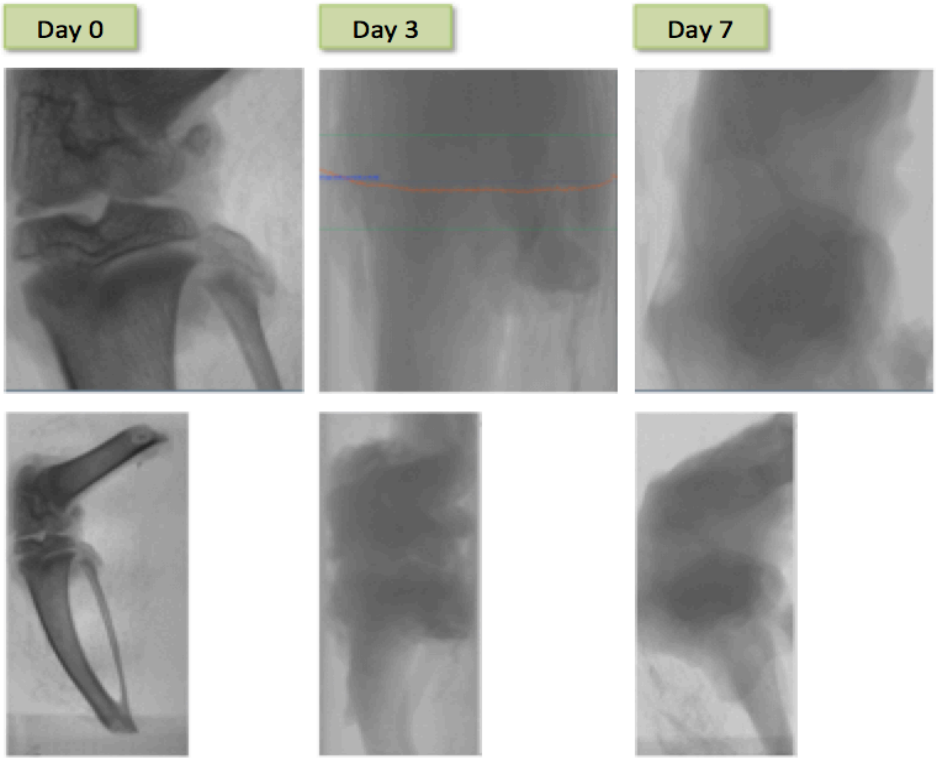


Figure 2.7 microCT images of the joint decalcifying over time. The day before decalcification begins (Day 0) the knee joint was scanned, and had obvious structure with clean edges. 3 days into the decalcification process the knee was scanned again, at this point the joint had lost a lot of structural integrity. After 7 days of decalcification in immunocal the joint had lost its structural integrity and had no calcium remaining on the bone.

2.5.2 Processing, embedding and cutting

Following decalcification the knees were removed from Immunocal® and rinsed in water before being dehydrated in increasing concentrations of ethanol. The decalcified knees were placed in 30% ethanol for 30 minutes, then 50% ethanol for 30 minutes and were finally placed in 70% ethanol. The knees were then placed in individual cassettes for overnight processing.

The processing protocol involved a series of ethanol and wax washes in a Tissue-Tek® VIPTM (Vaccum Infiltration Processor, Sakura, USA). Firstly, the knee joints were washed in 70% Industrial Methylated Spirits (IMS) for one hour. This was followed by a 90% IMS wash for one hour, followed by 4 100% IMS washes each at 90 minutes. Next, there were two xylene washes for 90 minutes, followed by two more xylene washes at 105 minutes each. The final step was a wax wash for 1 hour followed by two wax and vacuum steps each for 1 hour.

Upon the final stage of overnight processing in the Tissue-Tek® VIPTM, the knees were

placed in liquid wax ready for embedding. The knees were placed into individual plastic moulds and submerged fully in paraffin wax. The orientation of the knee was important for sectioning; the patella was faced down with the tibia perpendicular to the front of the mould and the femur facing upwards out of the mould. This allowed for all condyles of the joint to be cut evenly (Figure 2.7).

The joint was then cut in the paraffin wax block into 10μM sections using a microtome (Microm HM 360). Four consecutive knee slices were placed on a superforst plus slide (ThermoFisher), approximately 80 slides were produced per knee with the first slide representing the dorsal part of the knee and the last slide the ventral part of the knee. The slides were then left to dry in a 37°C oven overnight.

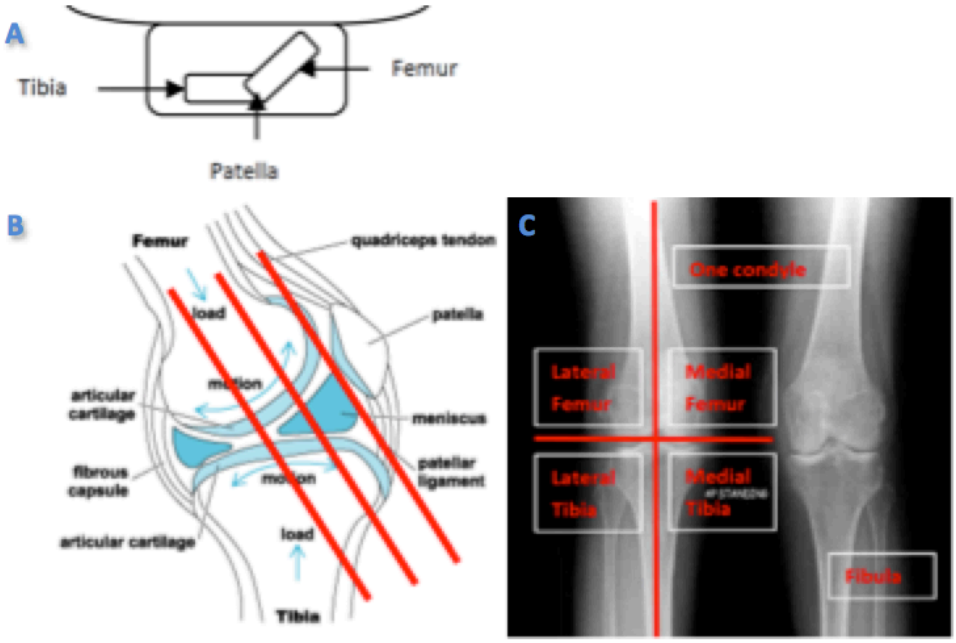


Figure 2.8 Orientation of the knee joint for sectioning. The knee joint was placed correctly in the mould ready for sectioning, such that all 4 condiles would be respresented in each knee section imaged.

2.5.3 Staining with Toluidine blue

Every fourth slide was stained to give an accurate representation of cartilage damage throughout the entire knee, I.e. slide 1, 5, 9, 13 etc was stained with toluidine blue. To begin with the slides underwent 2 washes with NeoClear, each for 10 minutes. The slides were then hydrated, starting with 2 100% ethanol washes at 1 minute each. The slides were then placed in 90% ethanol, followed by 70% ethanol, followed by 50% ethanol, followed by distilled water, for 1 minute at each concentration. The slides were then washed in acetate buffer for 1 minute before being placed in 1% toluidine blue

solution for 2 minutes. The slides were rinsed twice in distilled water until the water ran off clear. The slides were then placed into 2 acetone washes, both for 2 minutes each. The slides were then placed in xylene for 5 minutes and the placed into a second wash of fresh xylene for 2 minutes. There was a final fresh xylene wash before a cover slip (22mm x 70mm, 0.17mm thickness. Thermo Scientific) was mounted onto each slide with DPX.

2.5.4 Grading Lesions

Every fourth slide (1,5,9 etc.) was stained with toluidine blue to visualize cartilage and bone so that cartilage damage can be scored. Scoring was blinded and carried out by two individuals using a Zeiss light microscope with each condyle being scored separately. Knee scores were then calculated using two different methods. Firstly, the average maximum score was used where the highest scoring condyle was taken from each slide and averaged for all slides to represent damage throughout the knee. Secondly, the maximum score was used where the highest score through the entire knee was taken alone to represent the area of the most damage. The scoring system has been previously described (Glasson et al 2010); the scores are given as shown in Table 2.1 and Figure 2.8. The knee histology images presented in this thesis were produced using a Zeiss Axioskop microscope.

Table 2.1. Scoring system

Grade	Osteoarthritic damage
0	No damage
0.5	Some loss of toluidine blue staining
1	Small superficial lesions of the articular cartilage
2	Small lesions immediately below the superficial layer of articular cartilage
3	Large lesions or erosion of the articular cartilage covering up to 20% of the condyle
4	Loss of articular cartilage tissue from 20% to 50% of the condyle surface
5	Loss of articular cartilage tissue from 50% to 80% of the condyle surface
6	Loss of articular cartilage tissue from more than 80% of the condyle surface

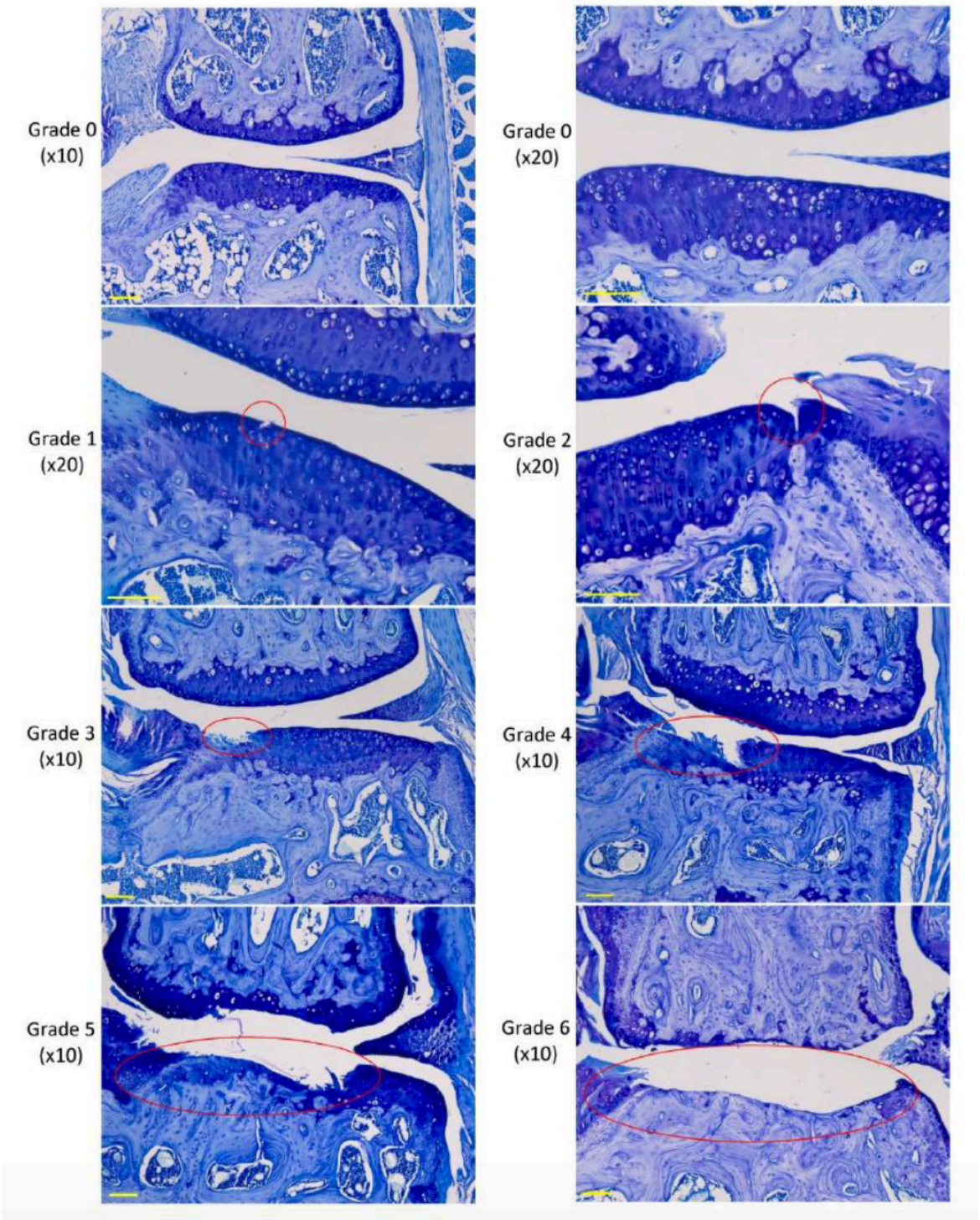


Figure 2.9. Sections of knee joints stained with toluidine blue showing the grades of articular cartilage lesions. The red circles show the area of damage and scores are determined based on the criteria set in Table 2.1.

2.6 Immunohistochemistry

2.6.1 Fast Blue retrograde neuronal tracer injection

Male Sprague-Dawley rats were anaesthetized with ketamine [0.6ml sterile saline, 0.1ml medetomidine hydrochloride (1mg/ml), 0.24ml ketamine (0.3mg/ml)], with 250μL injected interperitoneally per 100g of rat. The animals were checked to be fully unconscious and areflexive. The ventral surface of the hind limb was clipped of hair around the knee area and cleaned with clorhexidine. The joint was flexed in the left knee and an intra-articular injection of 25μL of Fast Blue (2% w/v in dH_2O)(Polysciences Europe GmbH) was adminstred with a 27g Hamilton needle through the infrapatellar ligament. The needle was held in place for 30 seconds and withdrawn slowly, and the animals remained unconscious for 2 hours to avoid movement and leakage of the Fast Blue. The animals were bought back to consciousness with the reversing agent atipamezole (5mg/ml), where 25μL was injected subcutaneously per 100g of animal.

2.6.2 Perfusion, dissection, embedding and slicing

Animals were terminally over-anaesthetised with an overdose of isoflourane and transcardially perfused, first with chilled 0.1% phosphate buffer to remove excess blood followed by 4% PFA for tissue fixation. The lumbar spinal cord was dissected out with the L3, L4 and L5 ipsilateral and contralateral DRGs. The tissue was placed in 4% PFA overnight for post-fixation. The tissue was then placed in 30% sucrose overnight before being mounted in OCT, frozen in liquid nitrogen and stored at -80°C.

DRGs were cut on a cryostat into 10μM sections and placed on SuperFrost Plus slides (ThermoFisher). Spinal cords were cut on a cryostat into 20μM sections and placed on SuperFrost Plus slides (ThermoFisher).

2.6.3 Antibody incubation and visualization

Slides were incubated with blocking buffer (10% donkey serum and 0.02% Triton-X-100 in PBS), for approximately 6 hours (over the day) at room temperature. Primary antibodies (βIII-tubulin - Invitrogen, mouse monoclonal, 1:1000, ATF-3 - Santa Cruz, rabbit polyclonal, 1:500) were incubated overnight at room temperature. Primary antibodies were removed by washing with PBS, 3 times (2 minutes each wash). Secondary antibodies conjugated with either Alexa-488 (anti-mouse) (Invitrogen), or Alexa-564 (anti-rabbit)(Invitrogen, 1:1000) were diluted in the blocking buffer

(1:1000), and slides were incubated for 2 hours at room temperature. Slides were washed 3 times in PBS (2 minutes each wash) and mounted in Fluoromount-G (ThermoFisher).

Immunofluorescence was visualized using a Zeiss Imager Z.1 microscope.

For Fast Blue retrograde neuronal tracer studies DRGs were taken for each lumbar section from 3 animals and 4 random slices of each DRG were chosen for images of complete DRG sections using mosaic-photomicrographs. The total numbers of βIII-tubulin nucleated neurons were counted in each DRG and their diameters measured using ImageJ. This was compared to the number of Fast Blue positive stained neurons so that the size distribution of the Fast Blue positive neurons and total population could be determined.

2.7 Quantitative Polymerase Chain Reaction (qPCR)

2.7.1 Perfusion and dissection

Animals were terminally over-anaesthetised with an overdose of Isofluorane and transcardially perfused with chilled 0.1% phosphate buffer to remove excess blood. The lumbar spinal cord was dissected and was sliced carefully down the central vessel to retrieve the ipsilateral side of the spinal cord and this was cut in half again to leave the ipsilateral dorsal horn. The L3, L4 and L5 DRGs were also dissected on both the ipsilateral and contralateral side. The tissue was then placed in RNAse free tubes and snap frozen using liquid nitrogen and stored at -80°C until RNA was extracted.

2.7.2 RNA extraction

Firstly the tissue is homogenized in Qiazol, chloroform is then added, the solution placed in Phase Lock Gel-Heavy (PLG) tubes and span in the centrifuge for 15 minutes at 12,000rpm at 4°C. The resulting aqueous phase from the centrifuge is poured onto RNAeasy spin columns (Qiagen RNAse microkit – Qiagen Germany) and processed according to the manufacturers instruction. In brief; 1.5 times the volume of ethanol was added to the spin column and span again at 11,000 rpm for 1 minute. After each spin the flow through is discarded. RW1 buffer was added and span at 13,000 rpm for 15 seconds, DNAse was then added and left to incubate at room temperature before spinning at 13,000 rpm for 15 seconds. The RW1 buffer step from above was repeated

and then this step was repeated with RPE buffer. Finally, 30μL RNAse free water was added onto the collection tube and the sample span at 11,000 rpm for 1 minute. The resulting RNA was immediately placed on ice and the concentrations determined using a Nanodrop Spectrophometer ND-100 (Labtech International, UK).

2.7.3 cDNA synthesis

First strand cDNA synthesis was performed with Superscript III Reverse Transcriptase (Invitrogen, Canada) according to manufacturers instruction. In brief, RNA was diluted to 500ng with RNAse free water made up to 12μL. To this solution, a 1μL aliquot of deoxynucleotide-triphosphate (dNTPs) was added containing equal amounts of adenine, guanine, thymine and cytosine (Promega, USA). Finally, a 1μL aliquot of random primers (50μM) was added (Promega, USA). This solution was then placed in a thermocycler (GeneAmp® PCR System 9700, Applied Biosciences) and heated to 65°C for 5 minutes before being placed back on ice. Secondly, a mastermix containing first strand buffer, dithiothreitol (DTT) and superscript III reverse transciptase was added to the RNA mixture (all Invitrogen, Canada). The entire sample is then placed back in the thermocycler using the following programme: 25°C for 5min, 50°C for 60min and 70°C for 15min. The cDNA was then stored at 4°C until qPCR was performed.

2.7.4 Primer validation

New primers were designed using primer-blast following pre-established criteria in our lab: product size between 70-150 base pairs, spanning exon-exon juntions, and minimal or no self-complimentary. Primers designed this way were IL-1β (InterLeukin-1β), HTR7 (5-HT_7-receptor), HTR3a (5-HT_{3A}-receptor), HTR3b (5-HT_{3B}-receptor), ADRA2 (α_2-adrenoceptor). In addition, ATF-3 (Activiating transcription factor 3), TNFα (tumour necrosis factor α), IL-6 (interleukin-6), GFAP (glial fibrillary acid protein), Cacna2d1 (the $\alpha_2\delta_1$ subunit of vaoltage gated calcium channels), AIF1 (Ionized calcium binding adaptor molecule 1), and GAPDH (Glyceraldehyde 3-phosphate dehydrogenase), which were also used in this study, had all been previously validated in the lab. The primer sequences used are shown in Table2.2.

Table 2.2 Primer sequences

Gene	Forward sequence (5'-3')	Reverse sequence (5'-3')	Source
IL-1β	AGGAGAGACAAGCAACGACA	TTTGGGATCCACACTCTCCAG	Invitrogen
HTR7	ACCTGAGGACCACCTATCGT	GGTCAGAGTTTTGTCTTACAGCA	Invitrogen
HTR3a	GGCTGCTAGATCACCTCCTG	AACCTGGTTCTTCTCATCCACG	Invitrogen
HTR3b	GTGGCTGTCGTAGGTATTCTAGG	CTGGTCTGACCTCCTTGTGG	Invitrogen
ADRA2	ACACGGACCTGCTTTGACAT	TATGCTGTTAGGCACAGGGG	Invitrogen
Ntrk1	TGTCAAGGCACTGAAGGAGAC	TGCAGACTCCAAAGAAGCGT	Invitrogen
AIF1	TCCCCACCTAAGGCCACCAGC	CGTCTCCTCGGACCACTGGA	Sigma
ATF-3	GGTCGCACTGACTTCTGAGG	CTCTGGCCGCTCTCTGGA	Sigma
TNFα	CGTCGTAGCAAACCACCAAGC	ATGGCGGAGAGGAGGCTGACT	Sigma
IL-6	TCTCTCCGCAAGAGACTTCC	CCGGACTTGTGAAGTAGGGA	Sigma
GFAP	CAACCTCCAGATCCGAGAA	TCTTGAGGTGGCCTTCTGAC	Sigma
Cacna2d1	CATTGTTGGGCTCCACAGTAT	GACCTTGTCACACTGGCAAA	Sigma
GAPDH	CTGCACCACCAACTGCTTAG	TGATGGCATGGACTGTGG	Sigma

For primer validation both rat spinal cord cDNA alone and a pool of rat cDNA containing heart, lung, spinal cord, muscle and liver were used. A 1:3 serial dilution of the cDNA was made with 5 concetrations; 12ng, 4ng, 1.33ng, 0.44ng and 0.15ng. RNAse free water was also used as a negative control. The exact protocol for qPCR is below. For each primer the serial dilution was used to create a standard curve, so the efficacy of the primer could be determined, as well as its melting curve. Furthermore, to confirm only one PCR product was being amplified, the products from the 4nd cDNA dilution were ran on electrophoresis 2% agarose gel (w/v TBE buffer, (PanReac AppliChem) containing Ethidium Bromie (Promega USA). To make the 2% gel 4g agarose (Sigma) was diluted in 200ml 10x TB buffer (PanReac AppliChem). Ethidium Bromide (14μL) was also added to the gel mixture, ethidium bromide binds to the DNA and allows us to visualize it using UV light. The set gel was placed in the electrophoresis unit and the unit was filled with 10x TB buffer until the gel was covered. After the qPCR reaction, 2μL loading buffer (Promega, USA) was added to each sample so that they could be loaded into wells within the gel. A 100 bp DNA ladder (Promega, USA) was loaded into the first well to verify the size of our PCR products. The gel was run at 100V for 1 hour so that the DNA products move towards the positive electrode at the bottom of the gel. The gel was imaged with UV light using GeneSnap (SynGene). The bands created by the PCR products were used to determine their molecular size and this is compared to the expected product size from the primers.

2.7.5 Quantitative Polymerase Chain Reactions

For the qPCR, an 8μL aliquot of master mix containing 5μL LightCycler® 480 SYBR Green I Master (Roche), 2μL RNAse free water, and 1μL (10μM) of the primers forward and reverse was prepared for each reaction. For each reaction , 2μL of 5ng/μL of cDNA was used. For all experiments, two technical replicates (duplicates) of each reaction were performed. A LightCycler® 384 well plate was placed in the LightCycler® 480 instrument (Roche), using a pre-optimised LightCycler® 480 SYBR Green I Master programe (for details, see Table2.3).

Table 2.3 LightCycler• 480 SYBR Green I Master Programme

Step	Target Temp (°C)	Hold (hh:mm:ss)	Ramp rate (°C/s)	Cycles	Analysis
Pre-Incubation	95	00:05:00	4.8	1	None
Amplification	95	00:00:10	4.8	45	Quantification
	72	00:00:30	4.8		
Melting Curve	95	00:00:05	4.8	1	Melting Curves
	65	00:01:00	2.5		
	97	-	-		
Cooling	40	00:00:10	2.0	1	None

2.7.6 Analysis

Using the analysis tab on the LightCycler® 480 programme, it was determined at which cycle the cDNA begins to be amplified into double stranded DNA products. When unbound, the SYBR Green dye exhibits very little fluorescence, however during the PCR, when double stranded DNA was produced the dye becomes intercalated into the DNA helix. The fluorescence of the SYBR green dye is greatly enhanced upon DNA binding. Therefore, an increase in the SYBR Green fluorescence is proportional to the amount of double-stranded DNA that has been produced; this is expressed as the Cp value (Figure 2.9). For all genes, expression was normalized to the expression of the housekeeper gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and this was expressed as the ΛCT value. Graphically, mRNA expression has been shown in two ways. Firstly, mRNA expression was expressed as the log of the ΛCT value. Secondly, the mRNA expression has been normalized relative to the Sham mRNA expression for comparison.

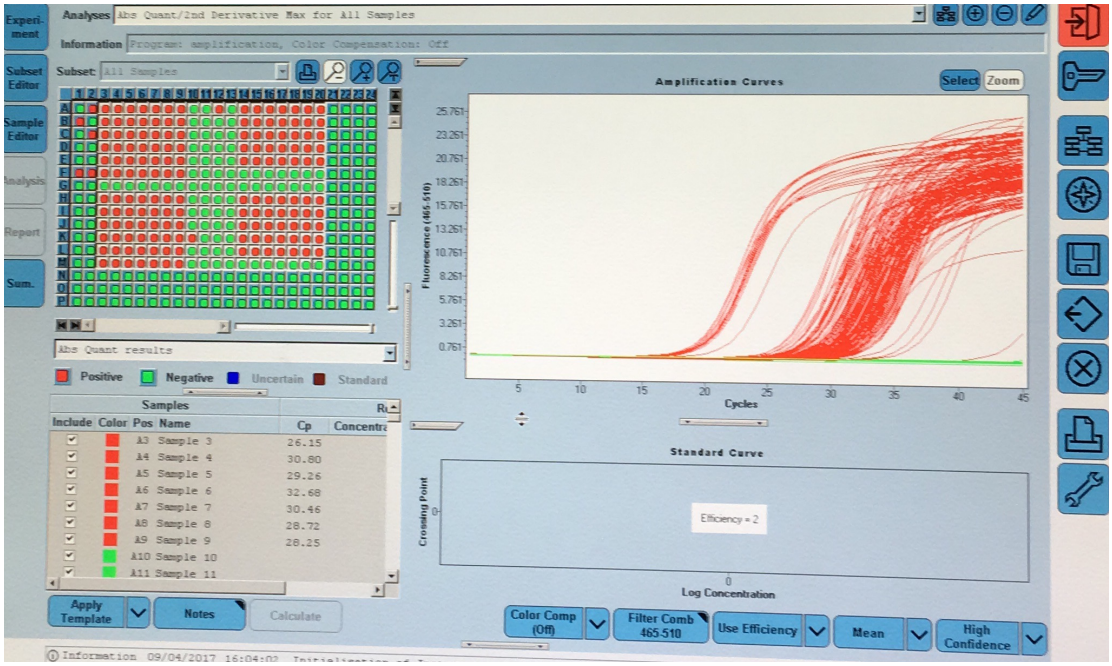


Figure 2.10 Analysis of qPCR results. The LightCycler® 480 programme uses 45 cycles of amplification. It determines at which cycle the cDNA begins to be amplified into double stranded DNA products resulting in an increase in SYBR Green fluorescence and this is expressed as the Cp value.

2.8 Statistical Analysis

All analysis was carried out with GraphPad Prism 6.0 for Mac OS X and SPSS Statistics version 22. Asterisks denote statistically significant differences (*P<0.05, **P<0.01, ***P<0.001).

2.8.1 Electrophysiology

Electrophysiological data is displayed as the number of action potentials fired ± the standard error of the mean (SEM).

The DNIC response was characterized as the number of action potentials fired when the receptive field was stimulated alone and compared to the number of action potentials fired with a concurrent noxious conditioning pinch. Statistical differences in neuronal firing following application of the noxious ear pinch were determined using a two-way repeated-measures analysis of variance (ANOVA) with a Bonferroni post-hoc test.

The drug time points plotted were the ones that produced the maximum change from the averaged pre-drug baseline values per animal. Statistical differences in the number of action potentials fired before and after drug administration were determined using a

two-way repeated-measures ANOVA with a Bonferroni post-hoc test. The DNIC percentages are determined as the inhibition of neuronal firing with concurrent application of the noxious ear pinch. The level of inhibition on neuronal firing and statistical differences between percentages following drug treatment in naïve animals were determined using a Kruskal-Wallis independent samples one-way ANOVA.

Statistical differences in mechanically evoked WDR neuronal responses between early and late phase MIA and Sham groups were tested using a one-way repeated-measures ANOVA with a Bonferroni correction.

2.8.2 Behavioural assessment of pain

Data showing mechanical hypersensitivity of the hind paw, representing secondary hyperalgesia, is presented as either the number of paw withdrawals (out of 5), the paw withdrawal threshold determined by the animal withdrawing 5 times to that von Frey hair, or as the 50% paw withdrawal threshold (PWT) derived from the up-down method as defined by Chaplan and colleagues (Chaplan et al 1994). For weight bearing, data is presented as the percentage of weight placed on the ipsilateral hind paw. All data is presented as the mean ± standard error of the mean (SEM). A Kurskall-wallis independent samples one-way ANOVA was used to test for differences in the number of withdrawals and paw withdrawal thresholds. A Freidmans two-way ANOVA by ranks was used to test for differences in the percentage weight distributed between ipsilateral and contralateral hind paws, while a Kruskall-wallis independent samples one-way ANOVA was used to test differences in the percentage of weight placed on the injected knee between MIA and Sham animals.

2.8.3 Knee histology

Knee histology scores were either presented as the maximum score where the highest score was taken through the entire knee or the average maximum score where the maximum score was taken from each slide and averaged through the knee. All the data was presented as the mean score ± SEM. A Friedmans two-way ANOVA by ranks test was used to determine statistical differences in knee histology scores between the four knee condyles. A Kruskall-wallis independent samples one-way ANOVA was used to test statistical differences in knee histology scores between MIA animals and sham controls.

2.8.4 Correlation between pain behaviour and knee histology score

Data was presented comparing either maximum knee histology score or average maximum knee histology score versus 50% PWT or the percentage of weight placed on the ipsilateral paw. Linear regression analysis was used to test for correlations between knee histology scores and pain-like behaviour in late phase MIA animals; when using this test R^2 represents the strength of the relationship between knee histology score and pain-like behaviour and an F test of overall significance determined if this relationship was significant.

2.8.5 Immunohistochemistry

Data was presented as the percentage of Fast Blue positive cell bodies in the DRGs ± SEM. A Freidmans two-way ANOVA with ranks test was used to test for statistical differences between the number of Fast Blue positive cell bodies between DRGs.

2.8.6 qPCR

All data represented mRNA expression levels relative to the housekeeping gene GAPDH, and the data was presented as either 2-ΔCT values or values relative to the sham control group. A one-way repeated-measures ANOVA with Bonferroni correction was used to test for differences in the mRNA expression between groups when data was expressed as 2-ΔCT values. A Kruskall-wallis independent samples one-way ANOVA was used to test for differences in mRNA expression between groups when the values were normalized to sham controls.

3. A model for investigating Diffuse Noxious Inhibitory Controls

3.1 Introduction

3.1.1 Assessing Diffuse Noxious Inhibitory Controls and Conditioned Pain Modulation

DNIC and CPM responses are mediated by final post-synaptic mechanisms acting on spinal convergent neurons (Villaneuva et al 1984a, Villaneuva et al 1984b). Consequently, there are three potential areas of study to investigate the function of these endogenous inhibitory controls further. Firstly, these controls of spinal neuronal activity and their pharmacology can be studied in animals using electrophysiology. Secondly, spinally mediated reflexes can be studied in both animals and humans. Thirdly, the perceived pain sensations to a testing stimulus in the presence of a noxious conditioning stimulus can be studied in humans. The combination of these methods has provided a lot of information on this endogenous descending inhibitory system (Yarnkitsky 2015).

3.1.2 Diffuse Noxious Inhibitory Controls can be observed in convergent neurons

The dorsal horn is an important site involved in processing sensory information and contains a diverse range of neurons (Todd 2010). Dorsal horn neurons can respond to a diverse range of stimuli depending upon the synapses they form with primary afferents. As previously discussed, WDR neurons are localized to the deep dorsal horn and receive input from Aβ, Aδ and C fibres meaning they respond to both innocuous and noxious peripheral stimulation. DNIC responses are observed in WDR neurons when a concurrent noxious stimulus is applied distant to the control stimulus, which activates top-down endogenous controls and results in powerful inhibition of all activities in WDR neurons (Le Bars et al 1979a). The Nociceptive Specific (NS) neurons are found predominantly in the superficial lamina of the dorsal horn, where they form synapses with Aδ and C afferents and so are specifically activated by noxious stimuli in the periphery (D'Mello and Dickenson 2008). Despite their role in the transmission of nociceptive information, NS neurons do not appear to be modulated by DNIC (Le Bars et al 1979b). The dorsal horn also contains proprioceptive neurons that respond to innocuous stimulation, they synapse only with Aβ primary afferents and therefore

respond to touch, light pressure, and some joint movement. The proprioceptive neurons cannot be activated by peripheral noxious stimulation within their receptive field, and their activity in response to innocuous stimulation appears to be unaffected by conditioning noxious stimulation outside of their receptive field (Le Bars et al 1979b). This suggests that DNIC specifically influences the activity of convergent neurons. Therefore, to assess the endogenous inhibitory actions of DNIC at the level of the spinal cord recordings must be taken from convergent neurons.

So long as the heterotopic noxious conditioning stimulus is noxious, a range of modalities can be used to activate a DNIC response. The activation of DNIC has been demonstrated with natural mechanical and thermal stimuli, through electrical stimulation of the periphery, and by more prolonged noxious stimulations such as chemical or visceral pain models (Bannister and Dickenson 2017). Electrophysiological recordings in the rat have demonstrated that the activity of convergent spinal neurons can be inhibited upon application of a concurrent noxious stimulation outside of the receptive field of the test stimulus, which includes immersion of the tail or the muzzle into a water bath heated within the noxious range, and a noxious mechanical pinch to the tail, ear or muzzle (Le Bars et al 1981, Dickenson et al 1981, Villanueva et al 1986). The actions of thermal conditioning stimulation have also been demonstrated in humans, where the thresholds required for activation of the nociceptive flexion spinal reflex mediated by electrical stimulation of the sural nerve were increased upon application of a concurrent heterotopic noxious thermal stimuli (Willer et al 1989). In rats, the neuronal activity of spinal convergent neurons evoked by electrical stimulation of C-fibres in the receptive field is inhibited by noxious conditioning stimuli (Villanueva et al 1986). Inflammatory pain models such as peripheral capsaicin injection, or intraperitoneal administration of acetic acid or bradykinin to induce visceral pain have also been used as the noxious conditioning stimulus to elicit a DNIC response (Calvino et al 1984, Dickenson et al 1981, Wiiting et al 1998, Itomi et al 2016). However this intense inflammation may lead to peripheral sensitization and may even mediate central neuronal changes. The model used to induce DNIC in this thesis needed to utilize a noxious conditioning stimulus that was short-lived and allowed for quick recovery without any risk of long-term neuronal changes.

3.1.3 Conditioned Pain modulation paradigms

There are multiple CPM paradigms that have been used in the clinic to measure the efficiency of the endogenous inhibitory system. CPM in human subjects allow for

assessment of changes in the perceived pain response upon application of a distant noxious stimulation, which when coupled with fMRI has allowed for brain areas that may be involved in the CPM process to be identified (Youseff et al 2015, Youseff et al 2016). Similarly to DNIC, a large range of innocuous and noxious stimulus modalities can be used as the test stimulus, but the conditioning stimulus must be noxious and at a distant body region. CPM paradigms often involve applying a test stimulus to the leg while simultaneously applying a conditioning stimulus to the opposite arm, or vice versa, and analyzing any changes to perceived pain thresholds. However, as there is no standardized protocol specifying the stimulus modality or duration of conditioning stimulus it can be difficult to compare findings from CPM studies (Pud et al 2009, Lewis et al 2012). Lewis et al found CPM responses to cold pressor and ischemic tests were reliable and consistent when repeated in the same session but the responses were more variable over multiple sessions (Lewis et al 2012). One problem with assessing perceived pain responses rather than directly assessing DNIC at the neuronal level is that the perceived pain is a complex response that can be influenced by a subject's mood, expectation, and their hormone and stress levels (Vincent and Tracey 2008, Lumley et al 2011). Furthermore, animal studies have shown that the inhibitory response of neurons can continue once the conditioning stimulus is removed, meaning a further discrepancy between CPM paradigms is when the inhibitory response is tested (Dickenson et al 1981, Villanueva et al 1986). As the duration of post-stimulus inhibition has not been conclusively determined, it is difficult to know if the CPM response finishes upon removal of the conditioning stimulus or once pain thresholds have returned to baseline (Lewis et al 2012). A standardized test for measuring pressure pain thresholds and a reliable paradigm for inducing inhibition with a conditioning noxious stimuli would allow for a more consistent comparison between CPM studies.

3.1.4 Chapter 3 aims

For this thesis, an anesthetized rat model was used to directly assess the inhibitory responses of convergent WDR neurons in the dorsal horn upon application of a noxious conditioning stimulus at a site distant to the receptive field.

3.2 Methods

3.2.1 Noxious conditioning stimulus placement

Pre-conditioned mechanically evoked WDR neuronal responses to 8g, 26g, and 60g stimulations on the hind paw receptive field were measured. A concurrent conditioning stimulus was then applied outside of the receptive field whilst stimulating the receptive field with the same von Frey hairs. The noxious conditioning stimuli was applied to the ipsilateral ear in most studies, but was also applied to the contralateral ear, the contralateral paw and the tip of the tail. All conditioning stimulations were induced with a bulldog serrefine clip (Interfocis, Linton, UK) and lasted for 5 seconds. Pre-conditioned and DNIC responses were averaged from two trials and DNIC was quantified as an inhibitory effect on neuronal firing during the concurrent noxious pinch.

3.2.2 The duration of DNIC induced neuronal inhibition

Two methods were used to assess how long the inhibitory effect on neuronal firing lasted once the concurrent conditioning stimulus had been removed.

Firstly, pre-conditioned mechanically evoked WDR neuronal responses to 8g, 26g, and 60g stimulations on the hind paw receptive field were measured. The receptive field was then stimulated with a concurrent noxious ear pinch for 5 seconds to obtain a DNIC response. The bulldog serrefine clip was then removed from the ear, and the receptive field was mechanically stimulated immediately after removal of the ear pinch and 10 seconds after removal of the ear pinch. DNIC was quantified as an inhibitory effect on neuronal firing compared to pre-conditioned neuronal responses. However, this only allowed a 5-10 second break between subsequent stimulations of the receptive field. Therefore, in a separate experiment I mechanically stimulated the receptor field, removed the von Frey hair and applied the same stimulation 5 seconds later. This was repeated with 8g, 26g and 60g von Frey hairs 3 times and a mean average was taken for number of action potentials fired to the first and second stimulus, to explore if this was an adequate time-frame for the WDR neuronal responses to return to baseline.

Due to the limitations described above a second method for measuring the duration of the DNIC response was used. Again, pre-conditioned mechanically evoked WDR neuronal responses to 8g, 26g, and 60g stimulations on the hind paw receptive field were measured. The receptive field was then stimulated with the same von Frey hairs

but with a concurrent noxious ear pinch for 5 seconds to obtain a DNIC response. In this instance the bulldog serrefine clip was then applied to the ear for 5 further minutes. The noxious ear pinch was then removed and the receptive field was mechanically stimulated 1 minute, 5 minutes and 10 minutes after. DNIC was quantified as an inhibitory effect on neuronal firing compared to pre-conditioned neuronal responses.

3.2.3 Statistical Analysis

For a description of the statistical tests used for analysis please refer to section 2.8.

3.3 Results

3.3.1 Diffuse Noxious Inhibitory Controls upon natural stimulation of the receptive field as the test stimulus

Electrophysiological recordings were taken from WDR neurons located in the dorsal horn at the depth expected (700-1000μM) for lamina IV-V of the dorsal horn, and received input from the hind paw. For mechanical stimulation, vonFrey hairs of increasing bending force, 8g, 26g, and 60g were applied to the receptive field 3 times to obtain a stable baseline response (<10% variation in action potential firing for all mechanically evoked neuronal responses). DNIC was characterized by mechanically stimulating the hind paw receptive field in the presence of a concurrent noxious ear pinch (Figure 3.1). For thermal stimulation, water jets heated to 42°C, 45°C, 48°C, and 50°C were applied to the receptive field twice to obtain a baseline response and DNIC was then induced during application of the thermal stimuli by a concurrent noxious ear pinch (Figure 3.1). Both mechanical and thermal DNIC trials were repeated twice and baseline and DNIC responses were averaged. The neuronal responses of WDR neurons were significantly inhibited to both mechanical and thermal stimuli upon application of the noxious ear pinch as shown in Figure 3.1. The concurrent noxious ear pinch consistently inhibited the WDR neuronal firing rate in response to both innocuous and noxious stimulation. Specifically, the levels of neuronal inhibition induced by a concurrent noxious ear pinch were 29%, 30% and 30% in response to 8g, 26g and 60g stimulations respectively. The levels of neuronal inhibition induced by a concurrent noxious ear pinch were 21%, 29%, 30% and 20% in response to 42°C, 45°C, 48°C, and 50°C stimulations respectively.

3.3.2 Diffuse Noxious Inhibitory Controls upon electrical stimulation of the receptive field as the test stimulus

WDR neuronal responses to electrical stimulation of the hind paw receptive field were taken. Specifically, the receptive field was stimulated with a train of 16 electrical pulses at 3 times the C-fibre threshold, and a post-stimulus time histogram (PSTH) was constructed to separate the responses evoked by peripheral afferent fibres based on their latency. The number of action potentials fired by Aβ, Aδ, and C fibres, as well as the number of action potentials fired due to post discharge, in response to the 16 electrical pulses was quantified.

A predicted no wind-up response was calculated and the hypothetical value was termed "input", which is what would be expected if the WDR neuron had no change in the evoked response with subsequent stimulations over the 16 pulse period. This allowed for a calculation of "wind-up" by taking the input value from the actual accumulative count of action potentials fired from the 16 stimulations.

The electrical stimulation of the receptive field was repeated 3 times, with a 10 minute rest period between each stimulation to allow for the neuronal response to return to baseline, and the results were averaged to obtain a pre-conditioned neuronal response. The electrical stimulation of the receptive field was then repeated but in the presence of a concurrent noxious ear pinch. Overall this counts as one DNIC trial, which is repeated twice and an average of the results were taken to obtain pre-conditioned and DNIC response values.

Interestingly, the noxious ear pinch appeared to exhibit no inhibitory effect on the electrically induced activity of the WDR neuron (Figure 3.2). There was no significant difference in the number of action potentials fired by the WDR neurons evoked by Aβ, Aδ, and C fibres with a concurrent noxious conditioning ear pinch. There was also no significant difference in the number of action potentials fired due to post-discharge of the neuron with the concurrent conditioning noxious ear pinch. Furthermore, the concurrent noxious ear pinch appeared to have no significant effect on the "wind-up" of the WDR neuron, as the number of action potentials fired increased by similar proportions with each subsequent electrical pulse in both the pre-conditioned and DNIC set-up.

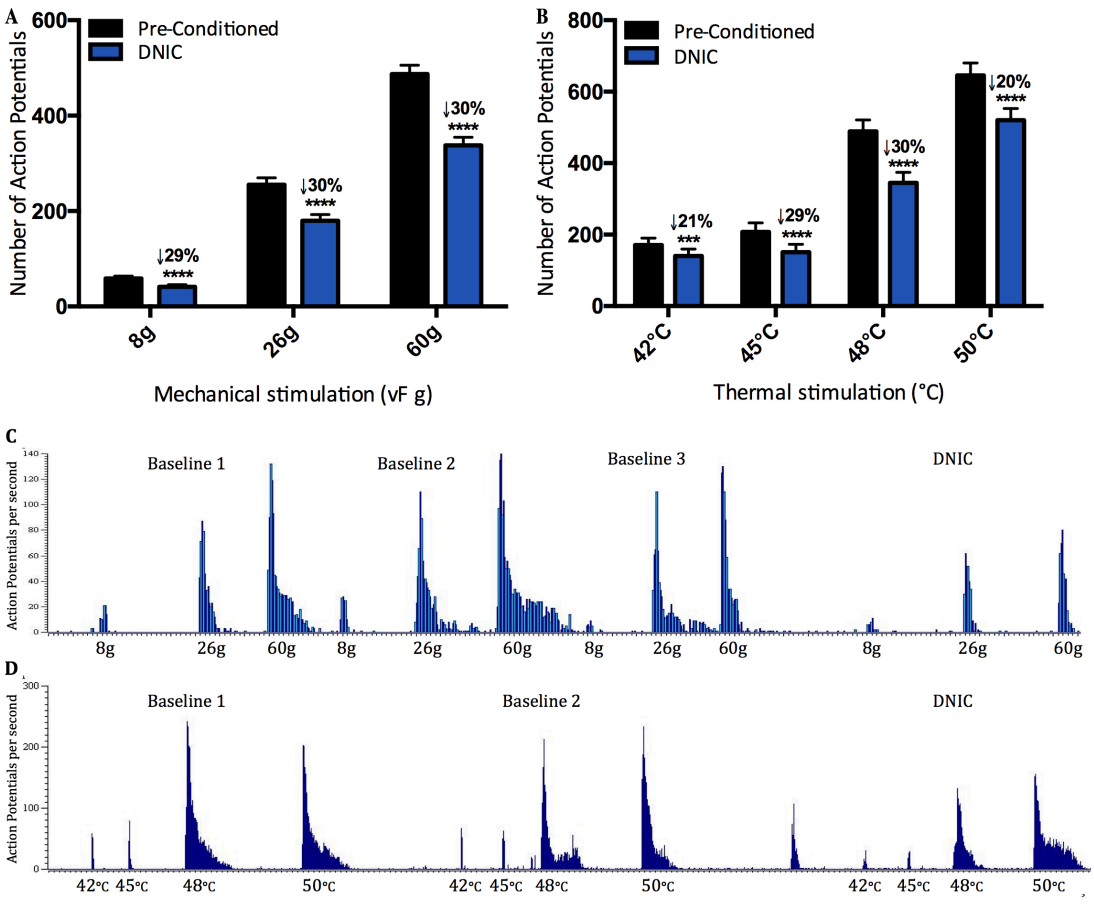


Figure 3.1. Mechanical and thermal stimulation of WDR neurons and the DNIC response. Electrophysiological recordings were taken from WDR neurons in the deep dorsal horn. A) The receptive field was mechanically stimulated with vonFrey hairs of increasing bending force (n=25). B) The receptive field was thermally stimulated by applying water jets of increasing temperatures (n=20). The mechanical and thermal stimulations were repeated to obtain baseline responses and DNIC was then induced with a concurrent noxious ear pinch. C) A raw trace from a single WDR neuron showing 3 stable baseline responses to von Frey hairs of increasing bending force and one DNIC response in the presence of a noxious conditioning ear pinch. D) A raw trace from a single WDR neuron stimulated twice to obtain stable thermal responses to water jets of increasing temperature followed by a DNIC response induced with a concurrent noxious ear pinch. Two-way ANOVA with Bonferroni post-hoc test (***P<0.001).

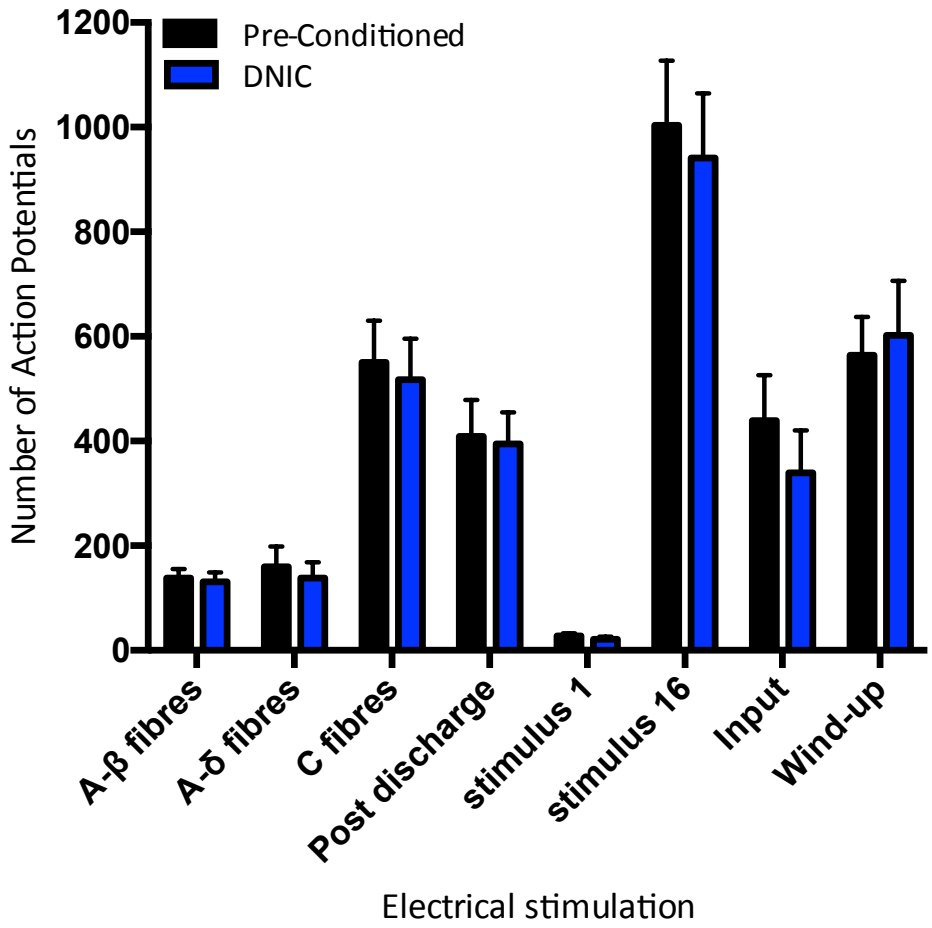


Figure 3.2. Electrical stimulation of WDR neurons and the DNIC response.
Electrophysiological recordings were taken from WDR neurons in the deep dorsal horn and the number of action potentials fired in response to stimulation of the receptive field with a train of 16 electrical pulses at 3 times the C-fibre threshold was quantified. The responses evoked by Aβ, Aδ, and C peripheral afferent fibres were quantified based on the latency of the responses. There was no significant difference in the number of action potentials fired by the WDR neurons due to activation of peripheral Aβ, Aδ, or C fibres with a concurrent noxious ear pinch. There was no significant difference in the "input" or "wind-up" responses of the WDR neurons in the presence of the noxious conditioning ear pinch. Two-way ANOVA with Bonferroni post-hoc test (n=6).

3.3.3 Placement of the conditioning noxious stimulus

It was evaluated how consistent the inhibitory response were when the concurrent conditioning noxious stimulus was applied to other areas of the body. The pre-conditioned mechanically evoked WDR neuronal responses were measured in response to 8g, 26g and 60g von Frey hairs. Whilst mechanically stimulating the receptive field a concurrent noxious pinch was applied at sites outside of the receptive field, this included the ipsilateral ear, the contralateral ear, the contralateral hind paw, and the tail. When the receptive field was mechanically stimulated with 8g and 26g von Frey hairs, there was a reduction in the number of action potentials fired upon application of conditioning noxious pinch to all areas, however this was only significant for the 8g

stimulation of the receptive field with noxious pinch of the contralateral paw (Figure 3.3). For mechanical stimulation of the receptive field with the 60g von Frey hair, the noxious conditioning pinch applied to all areas resulted in a significant inhibition of neuronal firing (Figure 3.3). This indicates that the noxious conditioning stimulus can be applied anywhere distant to the receptive field to produce an neuronal inhibition.

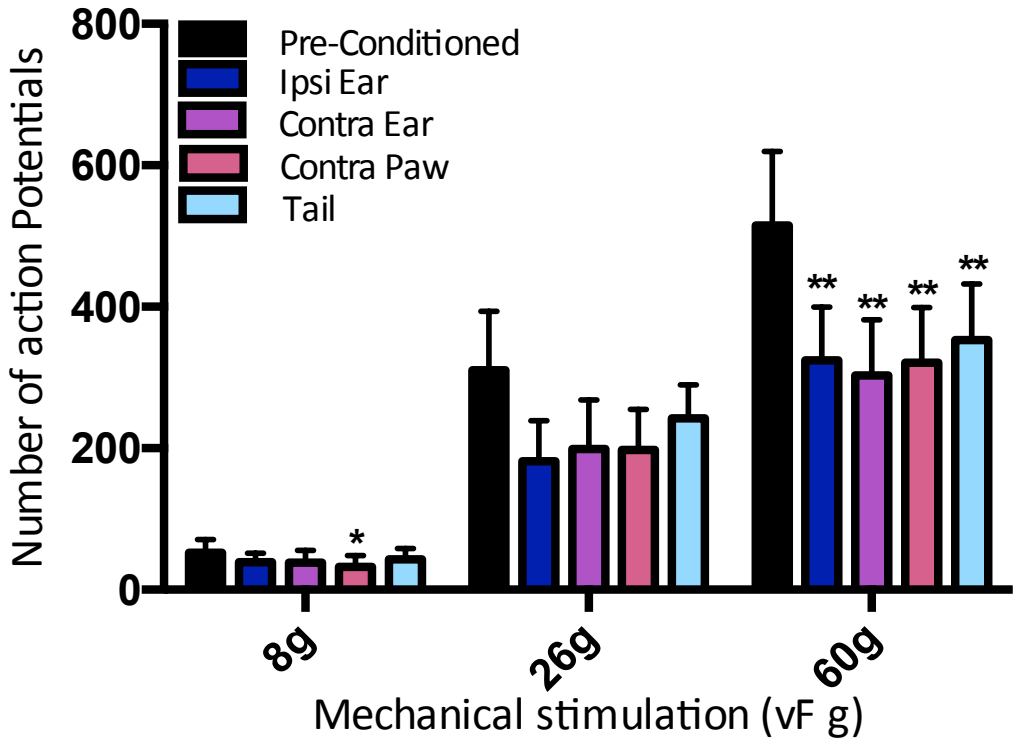


Figure 3.3 Placement of the noxious conditioning stimulus. Pre-conditioned neuronal responses to mechanical stimulation with 8g, 26g, and 60g von Frey hairs were measured from 3 stable baseline responses. The receptive field was then mechanically stimulated with the von Frey hairs but in the presence of a noxious pinch applied to the ipsilateral ear, contralateral ear, contralateral hind paw, and tail (n=6). The noxious pinch reduced the number of action potentials fired by WDR neurons in response to 8g and 26g stimulation at all locations but this was only significant for the 8g stimulation with the noxious pinch applied to the contralateral paw. For the most noxious mechanical stimulation of 60g, the number of action potentials fired by WDR neurons was significantly inhibited by the noxious conditioning pinch applied at all body areas. Two-way ANOVA with Bonferroni post-hoc test (*P<0.05, **P<0.01).

3.3.4 The duration of the neuronal inhibition following removal of the noxious conditioning stimulus.

Previous electrophysiological studies in the rat have demonstrated that a concurrent noxious conditioning stimulus causes strong post-stimulus inhibitory effects, yet the duration of these effects have not been quantified (Dickenson et al 1981, Villanueva et al 1986). Therefore, the duration of the DNIC induced WDR neuronal inhibition was evaluated once the noxious conditioning ear pinch had been removed.

Firstly, the ear pinch was applied for 5 seconds and the DNIC was quantified as an inhibitory effect on neuronal firing compared to pre-conditioned mechanically evoked neuronal responses. The concurrent noxious ear pinch was then removed and the receptive field mechanically stimulated with von Frey hairs immediately following removal of the noxious conditioning ear pinch and 10 seconds after removal. The number of action potentials fired by the WDR neuron following removal of the noxious conditioning ear pinch were counted and compared to pre-conditioned baselines. Similarly to the results observed in section 3.3.1, there was a significant inhibition of neuronal firing upon simultaneous mechanical stimulation of the receptive field in the presence of the conditioning noxious ear pinch (Figure 3.4A). The neuronal response was still inhibited immediately after removal of the noxious conditioning ear pinch but this was not significant for any mechanical weights tested (Figure 3.4A). When the receptive field was mechanically stimulated with von Frey hairs 10 seconds after the removal of the noxious conditioning stimulus, the neuronal responses had returned to pre-conditioned baseline levels (Figure 3.4A). However, this only allowed a few seconds between subsequent mechanical stimulations of the receptive field, so the effects of subsequent stimulations on neuronal responses were determined. The subsequent mechanical stimulation of the receptive field resulted in a higher number of action potentials fired by the WDR neuron than the original mechanical stimulation for all weights tested, and this was a significant increase for 8g and 26g (Figure 3.4B). This may suggest that the inhibitory response induced by the noxious conditioning stimulus is still ongoing but the effect is being masked by the sensitisation of the WDR neuron due to subsequent stimulations of the receptive field.

Secondly, the noxious ear pinch was applied for 5 minutes and DNIC was quantified as the inhibitory effect on neuronal firing compared to pre-conditioned neuronal responses. There was a significant neuronal inhibition induced with the concurrent noxious conditioning ear pinch. The noxious conditioning ear pinch was then removed and the receptive field was mechanically stimulated with von Frey hairs of increasing bending force at 1, 5 and 10 minutes following removal. Neuronal inhibition was observed 1 minute and 5 minutes following removal of the noxious conditioning ear pinch, but this was only significant for the most noxious mechanical stimulation of 60g (Figure 3.4C). The neuronal responses had returned to pre-conditioned levels 10 minutes after removal of the conditioning noxious ear pinch (Figure 3.4D). This suggests the DNIC response has powerful post-stimulus inhibitory effects that may be dependent on the intensity and duration of the noxious conditioning stimulus.

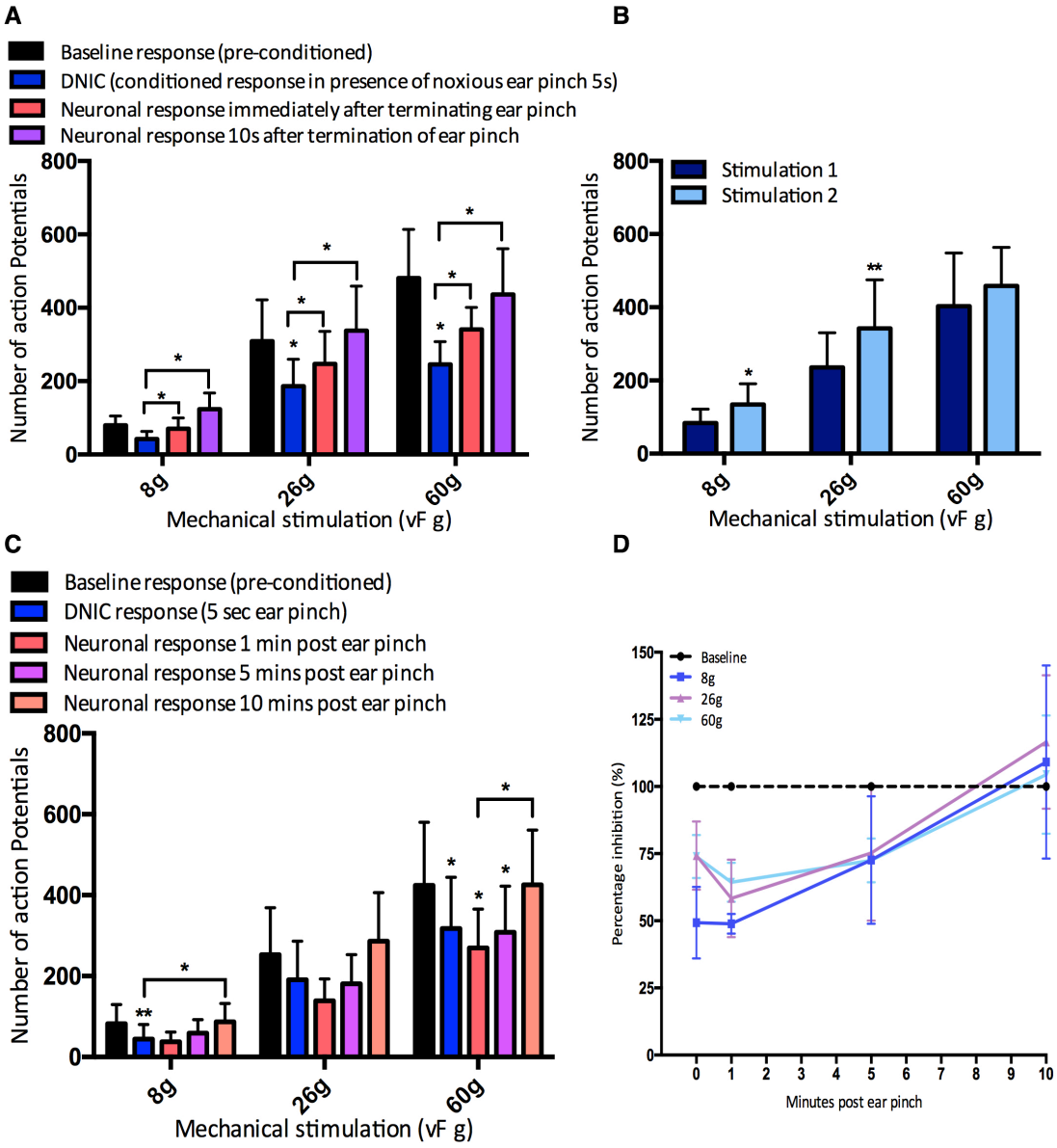


Figure 3.4. The duration of post-stimulus neuronal inhibition induced with DNIC. The duration of neuronal inhibition after removal of the conditioning noxious ear pinch was evaluated. A) The pre-conditioned neuronal responses to mechanical stimulation of the receptive field with von Frey hairs of increasing bending force were determined. Significant neuronal inhibition was induced by a concurrent noxious ear pinch. The neuronal responses were inhibited immediately after the removal of the noxious conditioning ear pinch but had returned to pre-conditioned baseline levels 10 seconds following removal of the noxious conditioning ear pinch (n=5). B) The subsequent mechanical stimulation of the receptive field with von Frey hairs directly after removal of the original mechanical stimulation caused WDR neurons to produce an increased number of action potentials (n=5). C) The noxious conditioning ear pinch was applied for 5 minutes. WDR neuronal firing was significantly reduced with a noxious conditioning ear pinch. The neuronal responses were also inhibited 1 minute and 5 minutes after removal of the noxious conditioning ear pinch but this was only significant when the receptive field was stimulated with 60g. At 10 minutes following removal of the conditioning noxious ear pinch the neuronal responses returned to pre-conditioned levels (n=6). D) This graph shows DNIC quantified as a percentage of the pre-conditioned values. With the concurrent noxious ear pinch and 1 minute following removal of the conditioning noxious ear pinch the neuronal responses

are approximately 60% of the pre-conditioned baseline values. At 5 minutes following removal of the noxious conditioning ear pinch the neuronal responses were approximately 75% of the pre-conditioned values for all weights tested. At 10 minutes following removal of the conditioning noxious ear pinch the neuronal responses returned to pre-conditioned levels (n=6). Two-way ANOVA with Bonferroni correction (*P<0.05, **P<0.01).

3.4 Discussion

DNIC represents the phenomenon whereby one pain inhibits another pain and relies on an endogenous inhibitory system. The model investigated in this chapter explores an electrophysiological method for exploring this phenomenon further. The model measures DNIC at the level of WDR convergent neurons located in the deep dorsal horn, and is quantified as an inhibitory effect on evoked neuronal firing. The noxious conditioning stimulus used in this model is a noxious mechanical pinch but different stimulus modalities were explored for use as the test stimulus. The noxious mechanical pinch used for the conditioning stimulus resulted in an inhibition of neuronal firing when applied to multiple body regions so long as it was distant from the test stimulus. Furthermore, the duration of the inhibitory response induced by the conditioning noxious pinch was explored at the neuronal level. This study reinforces the relevance and usefulness of studying WDR convergent neurons to analyse the DNIC phenomenon.

3.4.1 The use of different test stimulus modalities in the DNIC protocol

This study used recordings from WDR neurons in the deep dorsal horn receiving Aβ, Aδ and C fibre input from the hind paw. The first test stimulus tested was mechanical, where neuronal responses were evoked by applying von Frey hairs of increasing bending force to the receptive field on the hind paw. The weights used in the study were 8g, which is considered innocuous, 26g which is a noxious stimulation and would result in a withdrawal response behaviourally, and 60g which is a supra threshold stimulation at the highest end of the noxious range. The application of von Frey hairs heavier than 60g was not used as this may begin to cause tissue damage. The noxious conditioning ear pinch resulted in an inhibition of neuronal firing in response to all mechanical stimulations, indicating that DNIC is capable of inhibiting responses to both innocuous and noxious stimuli.

The DNIC response was then explored with a thermal test stimulus and a concurrent noxious conditioning ear pinch. Thermal stimulation of the WDR neuron was induced by applying water jets (42°C, 45°C , 48°C , and 50°C) to the receptive field of the hind paw. The thermo-TRP channels in the periphery are responsible for transducing thermal stimuli and can be activated over a range of both innocuous and noxious stimuli (Tominaga and Caterina 2004). The TRPV1 channel is the first receptor to detect noxious heat and has an activation threshold of >43°C, therefore the 42°C may be considered an innocuous thermal stimulation (Dhaka et al 2006). The 45°C thermal

stimulation is at the lower end of the noxious range while the 48°C and 50°C stimulations are noxious. None of the stimulations will activate the TRPV2 channels that do not become activated until temperatures of 52°C (Caterina et al 1999). Applying the noxious conditioning ear pinch resulted in an inhibition of neuronal firing in response to all thermal test stimulations, again suggesting that the concurrent noxious conditioning stimulation results in inhibition of both innocuous and noxious test stimulations.

Finally the DNIC response was assessed when the receptive field was electrically stimulated with a train of 16 electrical pulses at 3 times the C-fibre threshold, with a concurrent noxious conditioning ear pinch. The WDR neurons form synapses with Aβ, Aδ and C fibres that arise in the periphery and terminate in the dorsal horn, and the number of action potentials fired by the WDR neuron due to activation of these peripheral afferent fibres can be quantified by separating the responses based on their latency (Urch and Dickenson 2003, D'Mello and Dickenson 2008). Interestingly, the application of the conditioning noxious ear pinch had no inhibitory effect on the number of action potentials evoked by Aβ, Aδ or C fibres, or on the post-discharge of the neuron. Wind-up refers to the enhanced output of spinal cord neurons in response to rapid and repeated noxious stimuli, this can be observed when electrically stimulating the cell at 3 times the C fibre threshold as an increase in the number of action potentials fired in response to each subsequent electrical pulse in the train of 16. The noxious conditioning ear pinch did not inhibit the wind-up response of WDR neurons.

These results conflict with previous findings that A and C fibre activities of both trigeminal and dorsal horn convergent neurons evoked electrically are strongly inhibited by DNIC (Dickenson et al 1980, Bouhassira et al 1993). However, Bouhassira et al electrically stimulated the receptive field at the lower intensity of 2 times the C-fibre threshold rather than 3 times as was used in this study (Bouhassira et al 1993). Additionally a thermal noxious conditioning stimulus was used in both studies, induced by immersion of either the tail or muzzle in 50°C heated water. A further method used by Dickenson et al 1980 for inducing a noxious conditioning stimulus was the intraperitoneal administration of bradykinin, causing visceral inflammation (Dickenson et al 1980). The 50°C thermal stimulation used is at the higher end of the noxious range, while interperitoneal bradykinin would cause extreme visceral tissue damage, thus eliciting a more intense noxious conditioning stimulation than the use of noxious ear pinch. Dickenson et al did also show a DNIC induced neuronal inhibition elicited by applying a noxious pinch as the conditioning stimulus but they do not explain how this

was applied. Therefore, this was potentially of greater strength than the noxious ear pinch used in this study (Dickenson et al 1980). Therefore, the level of neuronal inhibition may be dependent upon the intensity of the conditioning stimulus.

The DNIC system functions through the conditioning noxious stimulus activating descending inhibitory controls, which reduces excessive pain through a negative feedback loop and allows the body to focus on the most dangerous stimuli (Le Bars et al 1979a). Three times the C fibre threshold has been used in these experiments to ensure the WDR neurons exhibit a wind-up response. A noxious ear pinch was used as the condition stimulus to elicit a short lived noxious stimulation that would not result in further problems such as tissue damage, but also for consistency as a noxious ear pinch has been previously show to produce a sufficient inhibitory response on neuronal firing (Bannister et al 2015). It has been reported that the magnitude of the DNIC response depends upon the intensity and stimulus modality used as the noxious conditioning stimulus (Fujii et al 2006). In this instance the noxious intensity of the electrical stimulation may be much greater than the conditioning ear pinch, this may cause the test stimulus to out-weight the conditioning stimulus meaning although the conditioning stimulus is noxious, it is not of adequate intensity to elicit an inhibitory effect on electrically evoked neuronal firing.

3.4.2 Placement of the conditioning noxious stimulus

This study demonstrated that mechanically evoked WDR neuronal activity can be significantly inhibited upon the application of a concurrent noxious conditioning pinch applied to widespread areas of the body. This indicates that descending inhibitory controls can be activated when the noxious conditioning stimulus is applied both ipsilaterally and contralaterally to the test stimulus. There are many methods used for evoking CPM in healthy subjects that have involved applying various stimulus modalities to various body regions, although the most common CPM paradigm uses a thermal or cold conditioning stimulus applied to the limbs or upper extremities (Pud et al 2009). The study confirms the validity of these studies for assessing CPM in humans as it demonstrates that a DNIC effect can be evoked by a noxious conditioning stimulus at multiple body regions distant to the test stimulus.

3.4.3 The duration of neuronal inhibition

This study demonstrated that the application of a noxious conditioning ear pinch had strong inhibitory post-stimulus effects on mechanically evoked WDR neuronal firing. The duration of the inhibitory effects following removal of the noxious conditioning stimulus appeared to be related to the duration the noxious conditioning stimulus was applied for. However, the duration of post-stimulus inhibitory effects may also be influenced by other factors such as the intensity, placement or stimulus modality used for the noxious conditioning stimulus as the duration of inhibitory effects observed varied greatly between studies (Lewis et al 2012). The findings in this study are consistent with other electrophysiological studies that quantify the DNIC effects as a reduction in convergent neuron firing, where powerful inhibitory were also observed after removal of the noxious conditioning stimulus (Dickenson et al 1981, Cadden 1993).

The use of fMRI in CPM paradigms has allowed for the identification of activated brain areas during the CPM process (Youseff et al 2015, Youseff et al 2016). However, as a subject is required to stay still in the fMRI machine to obtain good images, one of the problems with this set-up is applying two separate stimulations to the body while the subject stays still and inside the machine. Therefore, the finding that the noxious conditioning stimulus has powerful inhibitory effects even once it has been removed may have important clinical consequences. Potentially, the test and conditioning stimulations could be applied before the subject enters the fMRI machine, if the noxious conditioning stimulus is of the optimum intensity and quality the ongoing inhibitory effects may allow for activated brain areas to be identified shortly after the stimulus has been removed.

3.4.4 Concluding remarks

This chapter confirms that DNIC can be assessed using electrophysiological recordings from convergent WDR neurons in the deep dorsal horn. In this DNIC set-up the mechanically and thermally evoked WDR neuronal responses were strongly and consistently inhibited by the application of a concurrent noxious conditioning pinch. The noxious conditioning ear pinch did not result in an inhibition of WDR neuronal firing when the receptive field was electrically stimulated, therefore electrical stimulations is not used beyond this point. Pud et al reviewed the commonly used experimental methods for evoking a CPM response in healthy subjects and found the average magnitude of the inhibitory effect on pain thresholds to be 29% across the studies (Pud

et al 2009). Interestingly, the noxious conditioning ear pinch consistently results in an approximately 30% reduction in mechanically and thermally evoked WDR neuronal firing rates. The comparable inhibitory magnitude coupled with the similar function and pharmacology observed between DNIC and CPM suggests that this model may provide a useful tool for forward translation studies with great clinical relevance.

This model can be used as a tool to better understand the mechanisms, neurotransmitters and pathways involved in the DNIC phenomenon. A further clinical application of this model is to evaluate DNIC responses in animal models of chronic pain to assess any central changes that may occur. Specifically in this thesis, the DNIC protocol is used to analyse any changes in descending inhibitions during the progression of an animal model of osteoarthritis.

4. The roles of Noradrenaline and Serotonin in Diffuse Noxious Inhibitory Controls

4.1 Introduction

4.1.1 Descending Controls

Descending controls originating in the midbrain and brainstem can exert top-down modulation of pain processing at the level of the spinal cord (Lumb 2014). Descending controls mediate nociceptive transmission predominantly through the release of monoamines. There is a serotonergic system originating mainly in the rostral ventromedial medulla (RVM) and a noradrenergic pathway originating from pontine regions of the brainstem (Bannister and Dickenson 2016b). Serotonergic and Noradrenergic axons terminate diffusely throughout the dorsal horn and release noradrenaline (NA) and 5-hydroxytryptamine (5-HT) as neurotransmitters, this can mediate both excitatory and inhibitory effects depending upon which receptor they activate (Todd 2010, Bannister and Dickenson 2016b). While some serotonergic and noradrenergic axons form synapses with other neurons within the dorsal horn the majority of their action occurs through volume transmission, whereby the monoaminergic neurotransmitters are released directly into the non-synaptic extracellular dorsal horn space and can activate nearby receptors (Todd 2010). The bi-directional role of the monoamines on pain processing means they can both facilitate or inhibit the pain response.

It has previously been demonstrated that diffuse noxious inhibitory controls (DNIC) depend upon a balance between the opposing receptor-mediated actions of NA and 5-HT (Bannister et al 2015). Additionally, both noradrenergic and serotonergic systems can become altered in chronic pain states while DNIC-effects are often lost. Taken together these findings indicate that dysregulated monoaminergic descending controls may play a major role in the absence of DNIC in chronic pain states.

4.1.2 The noradrenergic descending system

Noradrenaline (NA) is a catecholamine produced and released by both peripheral and central nerve terminals, the subsequent physiological effects of NA depend upon the

activation of noradrenergic receptor subtypes (Summers and McMartin 1993). There are two major classes of noradrenergic receptor: the α-adrenoceptors and β-adrenoceptors, which can be further classified into α_1, α_2, β_1, and β_2 subtypes (Molinoff 1984). The α_1-adrenoceptors are localized centrally in the CA3 regions and denate gyrus of the hippocampus, and can also be found peripherally in smooth muscle (Hoyer et al 1990, Summers and McMartin 1993). The α_2-adrenoceptor subtype has been identified in multiple brain areas, specifically the hypothalamus, cerebral cortex and brainstem (Boyajian and Leslie 1987). In addition the α_2-adrenoceptor subtype is located centrally within the spinal cord, high densities of α_2 binding sites have been shown in both rat and human spinal cords, and α_2-adrenoceptor mRNA was identified in cell bodies at all lamina levels of the dorsal horn (Unnerstall et al 1984, Smith et al 1995). The α_2-adrenoceptor can be divided further into three pharmacological subtypes: $\alpha_2A, \alpha_2B, \alpha_2C$. While the α_2A and α_2C subtypes are found mainly in the central nervous system, the α_2B subtype is found on vascular smooth muscle and associated with vasopressor effects (Giovannitti et al 2015). The β-adrenoceptors are highly expressed throughout the brain, with the β_1-adrenoceptors found in the cortex, hippocampus, and ventral gelatinosa, while the β_2-adrenoceptors have been identified in the cerebellum, thalamic nuclei, cerebral cortex and olfactory tubercle (Summers and McMartin 1993). The β-adrenoceptors are also thought to play vital physiological roles in the kidney, blood vessels, uterus and heart (Summers and Mcmartin 1993). As expression of the α_2-adrenoceptor subtype has been identified at high levels in the spinal cord, and DNIC is mediated by a final post-synaptic mechanism at the level of the spinal cord, this thesis shall focus specifically on the α_2-adrenoceptor.

The noradrenergic cell groups are dispersed through the brainstem and are classified as A1-A7, with the A5-A7 cell groups forming descending noradrenergic projections to the spinal cord (Kwiat and Basbaum 1992, Pertovaara 2006). The A6 cell group is also know as the locus coeruleus (LC), this brainstem area has prominent expression of α_2-adrenoceptors and was found to have the highest expression levels of α_{2A}-adrenoceptor mRNA in the brain, therefore the LC is thought to be the main source of central noradrenaline (Unnerstall et al 1984, Scheinin et al 1994). However, the noradrenergic descending system also originates in the catecholaminergic A5 and A7 cell groups located in the ventral and dorsolateral pons respectively (Petrovaara 2006). The A5 and A7 cell groups also form projections that innervate the PAG, which subsequently projects to higher cortical structures, indicating that these noradrenergic cell groups modulate nociception through both ascending and descending systems (Petrovaara

2006). Once the NA neurotransmitter is released from the brainstem it can travel along descending axons to carry out its effect at the level of the spinal cord. Therefore the influences from higher cortical structures impact upon the noradrenergic cell groups, and ultimately influence the pain response at the level of the spinal cord through noradrenergic neurotransmission.

The catecholamine receptors that mediate the actions of noradrenaline generally utilize a guanine nucleotide regulatory binding protein (G-protein), which initiate a cascade of downstream cellular signaling events upon activation (Pertovaara 2013). The α_2-adrenoceptor subclass is coupled specifically to a G_i protein, which inhibits the actions of andenylate cyclase upon activation, this in turn reduces the intracellular levels of cAMP, and the final overall physiological response is hyperpolarization of the neuron (Giovannitti et al 2015). In addition the activation of α_2-adrenoceptors has been demonstrated to directly modify the activity of ion channels, in particular Ca^{2+} and K^+ channels and Na^+/H^+ antiports (Pertovaara 2013).

In nociception, noradrenaline is known to have an antinociceptive effect through its interaction with α_2-adrenoceptors in the spinal cord (Cervero and Jenson 2006). The spinal administration of noradrenaline has been shown to suppress the noxious evoked activity of dorsal horn WDR neurons in cats, and while this suppressive effect can be replicated by α_2-adrenoceptor agonists, the spinal application of α_2-adrenoceptor antagonists has been demonstrated to attenuate this inhibition in both the cat and rat (Collins et al 1984, Pertovaara et al 1991, Fleetwood-Walker et al 1985). Taken together, these results suggest that the antinociceptive action of noradrenaline is mediated in the dorsal horn through activation of the α_2-adrenoceptor.

Noradrenaline is thought to suppress the evoked activity of spinal dorsal horn neurones through several mechanisms. Firstly, presynaptic inhibition is thought to occur through action of noradrenaline on central terminals of substance P containing nociceptive primary afferent fibres, whereby noradrenaline acts to reduce noxious stimuli-induced excitatory postsynaptic potentials and inhibits the release of excitatory neurotransmitters such as glutamate and substance P, this subsequently prevents the transmission of nociceptive inputs to the dorsal horn (Stone et al 1998). There is also thought to be a postsynaptic element to noradrenaline's actions, which is mediated by the activation of Gi and second messenger cascades. As discussed above the inhibition of cAMP results in hyperpolarization of the neuron, this occurs due to an efflux of K+

through calcium-activated potassium channels, which subsequently prevents the influx of Ca^{2+} and suppresses neuronal firing (Giovannitti et al 2015). The hyperpolarization of substantia gelantinosa neurons, found in lamina II of the dorsal horn has been demonstrated upon perfusion of NA onto the spinal cord surface with in vivo whole-cell patch clamping (Sonohanata et al 2004). The hyperpolarization of dorsal horn neurons and the prevention of action potential firing inhibits the release of noradrenaline from these neurons into the dorsal horn, this subsequently prevents the activation of noradrenergic ascending projects, thus creating a negative feedback loop between the spinal cord and the brain (Giovannitti et al 2015). This negative feedback loop has been demonstrated to involve neurones of the spinothalamic tract, further confirming that noradrenaline is capable of modulating both ascending and descending pain signals (Westlund et a 1990). Finally α_2c-adrenoceptors are located on axon terminals of spinal excitatory interneurons, indicating that noradrenaline may activate and hyperpolarize these neurons to further inhibit the pain response at the level of the spinal cord (Olave and Maxwell 2004). The multiple mechanisms of action indicate the importance of noradrenaline in central pain modulation.

The noradrenergic system forms a prominent descending inhibitory pathway originating in the brainstem that often becomes altered in chronic pain states (Bannister et al 2009). The abundance of noradrenaline in the central nervous system and its multiple modes of action indicate an important system that could potentially be pharmacologically manipulated to provide efficient therapy of chronic pain.

4.1.3 The serotonergic descending system

As the actions of monoamines released from descending projections are mediated through volume transmission, the distribution of receptor subtypes within the dorsal horn largely determine their physiological action (Todd 2010). There are a myriad of receptors within the dorsal horn that 5-HT can act upon, meaning the roles of 5-HT in nociception are much more complex and difficult to determine (Bannister and Dickenson 2016b).

The descending serotonergic pathway arises in the RVM, which is a key brainstem structure responsible for relaying processed pain information from higher cortical structures to the spinal dorsal horn (Cai et al 2014, Bannister and Dickenson 2016b). The serotonergic neurones are located mainly in the nucleus raphe magnus (NRM) of

the RVM and become activated in response to noxious stimulation, this evokes the release of 5-HT in the spinal cord (Dogrul et al 2009). The descending serotonergic system exerts a bi-directional role on pain processing as both inhibitory and facilitatory actions have been demonstrated upon 5-HT release in the dorsal horn, this is thought to depend upon the receptor subtype activated and the physiological pain state of the subject (Porecca et al 2002, Gebhart 2004, Vanegas and Schaible 2004).

4.1.3.1 The 5-HT receptors

There are seven families of 5-HT receptors that together comprise at least fourteen pharmacologically and structurally distinct receptors subtypes that vary in their localization, coupling and ligand binding properties (Hoyer et al 1994, Barnes and Sharp 1999). The majority of the 5-HT receptor subtypes are G-protein coupled receptors (GPCRs), with the exception of the 5-HT_3 subtype which is a cation selective ion channel (Barnes and Sharp 1999).

5-HT_1 receptor

The 5-HT_1 family is made up of five subtypes: the 5-$HT_{1A,}$ 5-$HT_{1B,}$ 5-$HT_{1D,}$ 5-HT_{1E}, and 5-HT_{1F} receptors (Barnes and Sharp 1999). All 5-HT_1 receptor subtypes are reported to be coupled negatively to adenylate cyclase via G_i proteins, and can mediate the hyperpolarization of the neurons through the opening of K^+ channels, indicating they are likely to be involved in antinociception (Barnes and Sharp 1999). Particularly, the 5-HT_{1A} receptor subtype is found in high densities in superficial lamina of the dorsal horn and its agonists have been shown to block formalin-induced nociceptive responses, indicating that 5-HT can suppress nociception at the level of the spinal cord through activation of the 5-HT_{1A} receptor (Oyama et al 1996).

5-HT_2 receptor

There are three 5-HT_2 receptor subtypes, the 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors, all with similar signaling properties (Barnes and Sharp 1999, Rahman et al 2011). All three receptor subtypes are coupled positively to PLC via G_q proteins, their activation lead to increased accumulation of intracellular IP_3 and Ca^{2+}, these intracellular signaling events regulate neuronal excitability (Barnes and Sharp 1999, Billups et al 2006). The roles played by this receptor family in nociception are controversial with studies showing both antinociceptive and pronociceptive actions (Bardin et al 2000, Kjorsvik et al 2001, Rahman et al 2011). The intrathecal administration of 5-$HT_{2A/C}$ receptor agonists

immediately before the formalin test increased both early and late phase pain behaviours in the rat, indicating a pronociceptive role for this receptor (Kjorsvik et al 2001). In agreement, rat electrophysiological studies showed that a spinally administered 5-$HT2_A$ receptor antagonist dose dependently inhibited the evoked responses of WDR neurons to noxious mechanical and thermal stimuli, suggesting in normal conditions 5-HT_2 receptors facilitate spinal nociceptive transmission (Rahman et al 2011). However, the intrathecal pretreatment with both 5-HT_{2C} and 5-HT_{2A} receptor antagonists reversed the antinociception produced by intrathecal 5-HT alone in rats, indicating that increased levels of 5-HT were mediating antinociception through activation of these spinal 5-HT_2 receptors (Bardin et al 2000). These conflicting findings mean a facilitatory or inhibitory role for this receptor in nociception has not been conclusively determined.

5-HT_3 receptor

Unlike the controversial roles in nociception of other 5-HT receptors, the 5-HT_3 receptor has been clearly demonstrated to play a role in pronociception at the level of the spinal cord, particularly in chronic pain states (Green et al 2000, Suzuki et al 2002, Dogrul et al 2009, Kim et al 2015). In contrast to other 5-HT GPCRs, the 5-HT_3 receptor is a ligand gated ion channel that mediates a rapidly activating and desensitizing inward current carried predominantly by Na^+ and K^+ ions, but the channel is also permeable to Ca^{2+} (Barnes and Sharp 1999, Thompson and Lummis 2006).

One study confirming the involvement of the 5-HT_3 receptor in pronociception chemically interrupted the spino-bulbo-spinal loop, investigated the consequences and used pharmacology to unravel the receptors involved in facilitating the pain response at the level of the spinal cord (Suzuki et al 2002). The NK-1 receptor expressing projection neurons are found in superficial lamina of the dorsal horn and form part of an ascending pathways to the brainstem, which subsequently drives descending serotonergic inputs to the dorsal horn (Suzuki et al 2002). Suzuki and colleagues used a substance P-saporin (SP-SAP) conjugate to selectively ablate lamina I/III NK-1 expressing neurones within the spinal cord (Suzuki et al 2002). This treatment was found to reduce the excitability of WDR neurones and inhibit both mechanical and thermal evoked responses, while also attenuating the second phase of the formalin response (Suzuki et al 2002). Interestingly, the authors found spinal administration of a 5-HT_3 receptor antagonist ondansetron exactly mimicked this response in naïve and SAP injected animals, yet had no effect in the SP-SAP animals. The similar results observed with both SP-SAP treatment and

pharmacological blockade of 5-HT_3 receptors in the dorsal horn suggest both cause a reduction in descending excitatory pathways. The authors proposed that facilitatory descending projections arising in the RVM act on deep dorsal horn WDR neurones through 5-HT_3 receptors to create a pronociceptive drive (Suzuki et al 2002).

Furthermore, adaptive changes in the excitatory serotonergic drive mediating facilitation of the pain response through interactions with the 5-HT_3 receptors in the dorsal horn have been previously demonstrated in rat models of both osteoarthritis and SNL induced neuropathy (Bee and Dickenson 2008, Rahman et al 2009). This indicates that the activation of facilitatory descending controls and the subsequent release of serotonin within the dorsal horn may mediate an increase in the pain response at the level of the spinal cord through activating 5-HT_3 receptors, and that this may become over functional in states of chronic pain.

5-HT_4 and 5-HT_6 receptors

The 5-HT_4 and 5-HT_6 receptor families are both positively coupled to adenylate cyclase via G_s proteins. The activation of 5-HT_4 and 5-HT_6 receptors leads to increased intracellular levels of cAMP and subsequent activation of protein kinases, which can mediate phosphorylation of other channels or receptors expressed on the plasma membrane (Barnes and Sharp 1999, De Maeyer et al 2008). Specifically the activation of intracellular protein kinases can mediate the phosphorylation of K^+ channels and lead to their closure, which may suggest the activation of the 5-HT_4 and 5-HT_6 receptors is likely to result in depolarization and an increase in neuronal excitability (Barnes and Sharp 1999). Both receptor subtypes are expressed in the lumbar spinal cord and so are likely to become activated upon the release of 5-HT from descending pathways (Pineda-Farias et al 2017). The antinociception induced by intrathecal administration of 5-HT in the rat could not be alleviated by intrathecal administration of a 5-HT_4 receptor antagonist, indicating that this receptor was not involved in the antinociceptive actions of 5-HT at the level of the spinal cord (Bardin et al 2000). Furthermore, selective 5-HT_4 and 5-HT_6 receptor antagonists have been found to reduce allodynia in spinal nerve ligated rats, indicating a role for these receptors in facilitating the pain response (Pineda-Farias et al 2017). This suggests that spinal 5-HT_4 and 5-HT_6 receptors have a pronociceptive role and their activation may contribute to the maintenance of persistent pain.

5-HT$_5$ receptor

There are two subtypes of the 5-HT$_5$ receptor family, the 5-HT$_{5A}$ and 5-HT$_{5B}$ receptors (Nelson 2004). While both receptor subtypes have been found to be expressed in the rat and mouse, the 5-HT$_{5B}$ receptor is not expressed in humans due to stop codons interrupting the gene (Nelson 2004). The 5-HT$_5$ receptor is coupled to $G_{i/o}$ proteins, therefore its activation inhibits intracellular adenylate cyclase and mediates neuronal hyperpolarization (Nelson 2004). The use of specific antibody staining has shown particularly dense labeling for 5-HT$_5$ receptors in superficial layers of the dorsal horn suggesting this receptor may play a role in modulating nociception (Doly et al 2004). In rats with spinal nerve ligation, the intrathecal administration of 5-HT reversed the tactile allodynia caused by neuropathy, but an intrathecal 5-HT$_{5A}$ receptor antagonist reduced this antiallodynic effect (Avila-Rojas et al 2015). This indicates an antinociceptive role for this receptor and that its activation may inhibit pain processing at the level of the spinal cord.

5-HT$_7$ receptor

Finally, the 5-HT$_7$ receptor is a seven-transmembrane-domain GPCR positively coupled to adenylate cyclase (Hedlund and Sutcliffe 2004). As the 5-HT$_7$ receptor is linked to adenylate cyclase through a G_s protein, its activation results in increased intracellular levels of cAMP, this can initiate further downstream signaling events such as the activation of protein kinase A, and can also lead to the modulation of ion channels and receptors located in the plasma membrane (Barnes and Sharp 1999, Cooper 2000). While there are four splice variants of the 5-HT$_7$ receptor, only the 5-HT$_{7a}$, 5-HT$_{7b}$, and 5-HT$_{7c}$ variants appear to be expressed in rat and human tissue (Barnes and Sharp 1999). The 5-HT$_7$ receptor is expressed by umyleinated and thinly myelinated primary afferent fibres and pepetidergic neurons in the superficial lamina of the dorsal horn, making them likely candidates for mediating serotonins actions in pain control (Doly et al 2005).

The 5-HT$_7$ receptor has been demonstrated to play an antinociceptive role at the level of the spinal cord (Dogrul et al 2009, Brenchat et al 2010, Viguier et al 2012). Firstly, a spinally administered 5-HT$_7$ receptor antagonist SB-269970 reversed the antinocicepive actions of a morphine microinjection into the RVM in uninjured rats (Dogrul et al 2009). The authors proposed that opioidergic descending inhibitory projections arising in the RVM release 5-HT into the dorsal horn, which can subsequently activate 5-HT$_7$ receptors and suppress nociceptive transmission (Dogrul et al 2009). Additionally, systemic

administration of the selective 5-HT_7 receptor agonist AS-19 reduced the nerve-injury evoked mechanical and thermal hypersensitivity in mice, and this could be revered by the co-administration of the 5-HT_7 receptor antagonist SB-258719 (Brenchat et al 2009). Similarly, in rats with unilateral constriction injury to the sciatic nerve, subcutaneous administration of 5-HT_7 receptor agonists markedly reduced mechanical and thermal hyperalgesia (Viguier et al 2012). These studies indicate that activation of the 5-HT_7 receptor mediates inhibition of nociception at the level of the spinal cord in both naïve and neuropathic animals.

While the 5-HT_7 receptor shares molecular biology and activation properties with the 5-HT_4 and 5-HT_6 receptor, the 5-HT_7 receptor contrasts in that it mediates inhibitory actions in pain control at the level of the spinal cord. The different nociceptive actions could be attributed to distinctive neuronal localizations of these receptors. The 5-HT_7 receptor has been found to be co-localized with GABAergic cells in the dorsal horn, indicating the 5-HT_7 receptor is located on and plays a role in modulating the activity of inhibitory interneurons (Brenchat et al 2009). In agreement, an intrathecal injection of bicuculine, a competitive antagonist of GABA receptors, significantly reduced the anti-hyperalgesic effects of 5-HT_7 receptor activation with systemic administration of selective agonists in nerve injured rats (Viguier et al 2012). The activation of descending inhibitory projections arising in the RVM and the subsequent release of 5-HT in the dorsal horn may activate 5-HT_7 receptors to mediate excitation of GABAergic interneurons, which ultimately causes an inhibition of the pain response. This thesis will focus on the role played by the 5-HT_7 receptor in modulating the pain response at the level of the spinal cord, and particularly its role in mediating DNIC.

4.1.3.2 The modulation of the serotonergic system

In addition to the myriad of receptors that 5-HT can act upon in the dorsal horn, the serotonergic descending system becomes yet more complex due to the variation in its signaling observed in different physiological states. Following the depletion of 5-HT in the lumbar cord with 5,7-dihydroxytryptamine (5,7-DHT), the intrathecal administration of 5-HT was found to have a bi-directional effect on the second phase of the formalin response (Oyama et al 1996). The authors found that a low dose of 5-HT inhibited aversive responses and that this could be blocked by intrathecal administration of a 5-HT_{1A} receptor antagonist. Whereas, a high dose of 5-HT facilitated the aversive behaviours observed in the formalin test and this could be blocked by

intrathecal administration of a 5-HT_3 receptor antagonist. The authors concluded that the 5-HT receptor subtypes possessed different affinities for 5-HT and their activation was dependent upon the levels of 5-HT present in the dorsal horn (Oyama et al 1996). Similarly, the depletion of 5-HT in the RVM using a short-hairpin-RNA-interference to block the actions of the rate-limiting enzyme for the synthesis of 5-HT, tryptophan hydroxylase 2 (Tph-2), attenuated formalin-induced nocifensive behaviours during the second phase but had no effect in the first phase (Wei et al 2010). The authors suggest that release of 5-HT from descending projections arising in the RVM may not be involved in the initiation of acute pain but does play a role in the development and maintenance of persistent pain (Wei et al 2010). The bi-directional role of the RVM mediating both descending inhibitory and excitatory influences at the level of the dorsal horn may shift towards facilitation following tissue or nerve injury and this may be mediated by an alteration in the 5-HT receptors activated.

4.1.4 The use of noradrenaline and serotonin re-uptake inhibitors for the treatment of chronic pain

The main clinical symptoms associated with chronic pain are allodynia and hyperalgesia, whereby innocuous stimuli produce a painful response and responses to noxious stimuli become increased (Jensen and Finnerup 2014) (See Section 1.1.4.2). Chronic pain conditions, especially neuropathic pain, are becoming increasingly difficult to treat in the long-term with conventional analgesics such as opioids and NSAIDs, due to their limited effectiveness, adverse side effects, and patients developing tolerance (Foley 2003, Bomholt et al 2005, Ong et al 2007). A further potential option for analgesia is the use of drugs traditionally designed as antidepressants that function by modulating the levels of extracellular noradrenaline and serotonin (Bomholt et al 2005, Sindrup et al 2005). Tricyclic antidepressants inhibit the presynaptic reuptake of the monoamines NA and 5-HT but are also thought to work through other mechanisms involving the blockade of ion channels and NMDA receptors (Sindrup et al 2005). Similarly, selective serotonin reuptake inhibitors (SSRIs) and selective noradrenaline reuptake inhibitors (NRIs) function by specifically inhibiting the presynaptic reuptake of either serotonin or noradrenaline, while serotonin noradrenaline re-uptake inhibitors (SNRIs) combine this effect and prevent the reuptake of both monoamines from the synaptic space. Therefore, these drugs function by increasing the availability of NA and 5-HT at the synapse, which may act on α_2-adrenoceptors and/or 5-HT receptors in the dorsal horn to mediate inhibitory control of the pain response (Singh et al 2001).

Tricyclic antidepressants, SNRIs, and SSRIs have proved effective at relieving pain in patients with peripheral neuropathy (Sindrup et al 2005).

Along with the clinical symptoms of allodynia and hyperalgesia, chronic pain is often coupled with dysfunctional CPM or DNIC, which has been demonstrated in both human subjects and animal models respectively (Ardent-Nielsen et al 2008, Bannister et al 2015) (See Section 1.2.3). Duloxetine is a selective SNRI, and the efficacy of this drug in patients with painful diabetic neuropathy was found to be correlated with baseline CPM, such that patients with an ineffective CPM system pre-treatment would benefit the most from duloxetine treatment (Yarnitsky et al 2012). Additionally, Bannister et al have demonstrated that both spinal and systemic administration of the NRIs, Reboxetine and Tapentadol respectively, restored DNIC in a spinal nerve ligation rat model of neuropathy (Bannister et al 2015). These findings indicated that increased synaptic availability of 5-HT and NA may be sufficient to restore this endogenous inhibitory system, and may be one mechanism by which these drugs produce analgesia in patients.

4.1.5 Chapter 4 aims

In this chapter a tricyclic antidepressant, an NRI, and SSRIs are used to increase the systemic and spinal synaptic availability of noradrenaline and serotonin, and the subsequent effect on DNIC is investigated in both naïve and neuropathic animals. In the case of spinally administered SSRIs, when the synaptic content of 5-HT is dramatically increased, the 5-HT receptors activated were investigated.

4.2 Methods

4.2.1 Pharmacology

One pharmacological study was carried out per animal, using one WDR neuron. In SNL animals the WDR neuron was located in the ipsilateral dorsal horn relative to the nerve injury.

Naïve animals:

- **Reboxetine** (Tocris, Bristol, UK) is a noradrenaline reuptake inhibitor (NRI). Reboxetine was dissolved in vehicle (97% normal saline, 2% cremaphor, 1% DMSO) and 100µg/50µL was applied topically to the spinal cord. Neuronal responses were tested 20, 40 and 60 minutes after application.

- **Fluoxetine** (Sigma, Welwyn Garden City, UK) is a selective serotonin reuptake inhibitor (SSRI). Fluoxetine was dissolved in normal saline and applied both spinally (100µg/50µL) and systemically (20mg/kg). For the systemic dose fluoxetine was dissolved in normal saline and administered via a subcutaneous injection. The dose used was 20mg/kg, for this a 20mg/ml solution was made up and the amount injected was dependent on the weight of the animal. For example a 250g rat would receive 250µL of this solution. In both cases neuronal responses were tested 20, 40 and 60 minutes after administration.

- **Amitriptyline** (Tocris) is a tricyclic antidepressant that predominantly inhibits the reuptake of serotonin and noradrenaline. This was dissolved in normal saline and delivered via a subcutaneous injection at a dose of 5mg/kg. Neuronal Responses to the drug were followed over an hour with tests carried out at 10, 30 and 50 minutes.

SNL animals:

- **Fluoxetine** was dissolved in normal saline and applied spinally (100µg/50µL). A 20mg/kg systemic dose of fluoxetine was also used. For this fluoxetine was dissolved in normal saline and administered via a subcutaneous injection.

- **Citalopram** (Tocris) is also a selective serotonin reuptake inhibitor (SSRI). Citalopram was dissolved in normal saline and applied spinally (100µg/50µL). A systemic dose of citalopram was investigated, for this citalopram was dissolved in normal saline and administered via a subcutaneous injection with a dose of 10mg/kg.

- **SB-269970** (Tocris) is a selective 5-HT_7 receptor antagonist. This was dissolved in saline and applied spinally (100µg/50µL) in combination with fluoxetine or citalopram delivered at the same concentration.

- **Atipamezole** (Sigma) is an α_2-adrenoceptor antagonist. Atipamezole was dissolved in vehicle (97% normal saline, 2% cremaphor, 1% DMSO) and a concentration of (100µg/50µL) was applied topically to the spinal cord in combination with fluoxetine or citalopram at the same concentration.

For all experiments in SNLs, the neuronal response to drug was followed for one hour with tests carried out at the time points 20 and 40 minutes.

For all drug time points one DNIC trial was carried out involving 3 baseline pre-conditioned responses and one DNIC response in the presence of noxious ear pinch.

4.2.2 Statistical Analysis

For a description of the statistical tests used for analysis please see section 2.8.

4.3 Results

4.3.1 Spinal reboxetine in naïve animals

Throughout this chapter, DNIC was induced by a concurrent noxious ear pinch ipsilateral to the WDR neuron being recorded. The magnitude of inhibition produced by DNIC was investigated in naïve rats following the spinal application of the NRI reboxetine. For mechanical stimulation of 8g, 26g, and 60g the pre-treatment inhibition produced with DNIC were 26%, 36%, and 35% respectively. Following spinal administration of reboxetine, the inhibition of neuronal firing with DNIC was 41%, 50%, and 42% for mechanical stimulations of 8g, 26g, and 60g respectively (Figure 4.1a). Additionally, the spinal application of reboxetine significantly reduced the pre-conditioned mechanically evoked neuronal responses to 26g and 60g (Figure 4.1b). For thermal stimulation of 42°C, 45°C, 48°C, and 50°C the pre-treatment inhibition of neuronal firing produced with DNIC were 33%, 30%, 26%, and 17% respectively. Following spinal administration of reboxetine, the magnitude of inhibition produced by DNIC was 27%, 34%, 36%, and 27% for 42°C, 45°C, 48°C, and 50°C respectively (Figure 4.1c). The spinal application of reboxetine does not increase the magnitude of inhibition produced by DNIC to the thermal stimulations of 42°C and 45°C, but does increase the degree of inhibition produced by DNIC for the thermal stimulations higher in the noxious range. In addition, the spinal application of reboxetine significantly reduced the pre-conditioned thermal evoked neuronal responses to 45°C and 48°C (Figure 4.1d). Overall for all mechanically evoked responses and for the thermally evoked responses in the higher noxious range, the spinal application of reboxetine increases the magnitude of inhibition induced by DNIC by approximately 10%.

4.3.2 Systemic amitriptyline in naïve animals

The level of inhibition produced by DNIC was investigated in naïve rats following a subcutaneous injection of the tricyclic antidepressant amitriptyline. For pre-treatment mechanical stimulation, the inhibition of neuronal firing in response to 8g, 26g, and 60g was 27%, 25%, and 26% respectively. Following systemic administration of amitriptyline the magnitude of inhibition induced with DNIC was 42%, 34%, and 33% to 8g, 26g, and 60g respectively (Figure 4.2a). The systemic amitriptyline did not significantly affect the pre-conditioned mechanically evoked responses (Figure 4.2b). For thermally evoked neuronal responses, the inhibition induced by DNIC before amitriptyline treatment was 8%, 19%, 28%, and 20%, in response to 42°C, 45°C, 48°C,

and 50°C respectively. Following subcutaneous injection of amitriptyline the magnitude of inhibition induced by DNIC was 10%, 19%, 22%, and 15% in response to 42°C, 45°C, 48°C, and 50°C respectively (Figure 3.2c). While amitriptyline treatment reduced the pre-conditioned neuronal responses evoked by the more noxious thermal stimulations of 48°C and 50°C this effect was not significant (Figure 4.2d). Therefore, amitriptyline treatment increased the magnitude of inhibition produced by DNIC for mechanical stimulation but did not inhibit the pre-conditioned mechanically evoked neuronal responses. Additionally, amitriptyline treatment did not increase the magnitude of inhibition induced by DNIC in response to thermal stimulation or inhibit the pre-conditioned thermally evoked neuronal responses.

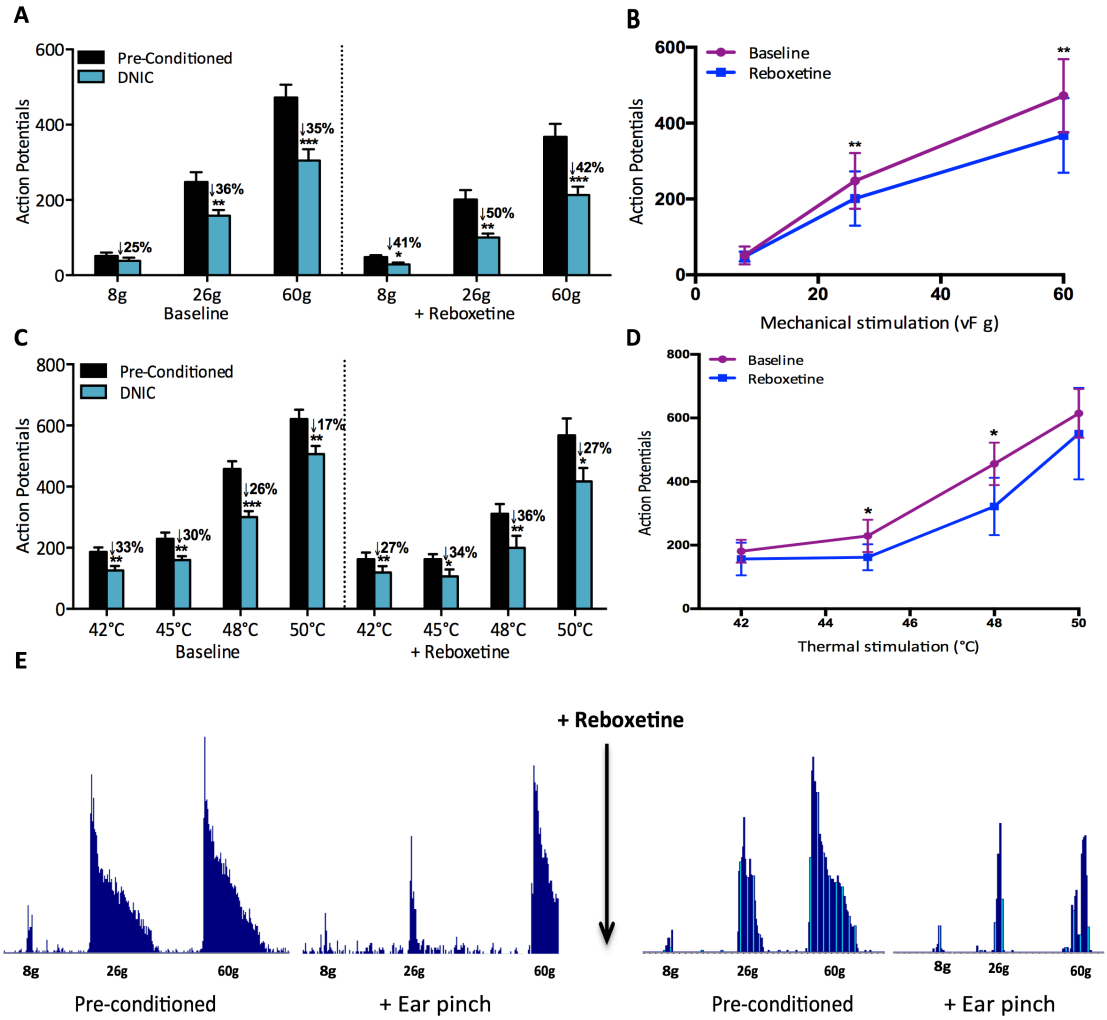


Figure 4.1. Spinal application of the NRI, reboxetine, in naïve animals. A) The mechanically evoked neuronal responses and the degree of inhibition induced by DNIC before and after spinal application of reboxetine (100µg/50µL) (n=8). B) The baseline pre-conditioned mechanically evoked neuronal responses were significantly inhibited for 26g and 60g following spinal administration of reboxetine. C) The thermally evoked neuronal responses and the magnitude of inhibition induced by DNIC before and after spinal application of reboxetine (100µg/50µL) (n=8). D) The baseline pre-conditioned thermally evoked neuronal responses were significantly

inhibited for 45°C and 48°C. E) A raw trace from one example WDR neuron, displaying one example pre-conditioned baseline response to vonFrey hairs of increasing bending force, followed by a pre-treatment DNIC response, the raw trace shows a concurrent reduction in neuronal firing upon application of the noxious conditioning ear pinch. Following spinal administration of reboxetine, one example pre-conditioned mechanically evoked response is shown to vonFrey hairs of increasing bending force, followed by a DNIC response induced by concurrent application of a noxious conditioning ear pinch. This raw trace displays how the mechanically evoked neuronal firing is inhibited to a greater magnitude following spinal application of reboxetine. Two-way ANOVA with Bonferroni post-hoc test (*P<0.05, **P<0.01, ***P<0.001).

4.3.3 Spinal and Systemic fluoxetine in naïve animals

The magnitude of inhibition on mechanical and thermal evoked neuronal responses induced by DNIC was investigated in naïve rats following both spinal and systemic administration of the SSRI Fluoxetine. For mechanically evoked responses before spinal Fluoxetine treatment the magnitude of inhibition induced by DNIC was 33%, 23%, and 30% for 8g, 26g and 60g respectively. Following spinal application of Fluoxetine the degree of inhibition induced by DNIC was 31%, 36%, and 24% to 8g, 26g, and 60g respectively (Figure 4.3a). The spinal application of Fluoxetine significantly inhibited the pre-conditioned mechanically evoked neuronal firing in response to 26g and 60g (Figure 4.3b). For mechanically evoked responses before systemic Fluoxetine treatment the magnitude of inhibition induced by DNIC was 44%, 34%, and 31% for 8g, 26g, and 60g respectively. Following subcutaneous injection of Fluoxetine the magnitude of inhibition induced by DNIC was 54%, 34%, and 27% to 8g, 26g, and 60g respectively (Figure 4.3c). The subcutaneous injection of Fluoxetine did not significantly affect the pre-conditioned mechanically evoked neuronal responses (Figure 4.3d). For mechanically evoked neuronal responses, neither the spinal or systemic administration of Fluoxetine consistently affected the degree of inhibition induced by DNIC. Additionally, the spinal application of Fluoxetine significantly reduced the mechanically evoked pre-conditioned neuronal responses while the systemic application of Fluoxetine have this effect. For thermally induced neuronal responses before spinal Fluoxetine treatment the degree of inhibition induced by DNIC was 11%, 32%, 26%, and 21% in response to 42°C, 45°C, 48°C, and 50°C respectively. Following the spinal administration of Fluoxetine, the degree of inhibition induced by DNIC was 20%, 24%, 25%, and 17% in response to 42°C, 45°C, 48°C, and 50°C respectively (Figure 4.3e). The spinal application of Fluoxetine significantly inhibited the thermally evoked pre-conditioned neuronal responses to 48°C and 50°C (Figure 4.3f). For thermally evoked neuronal responses the spinal application of Fluoxetine did not increase the magnitude

of inhibition induced by DNIC, but did significantly inhibit the pre-conditioned neuronal responses to thermal stimulations in the higher noxious range.

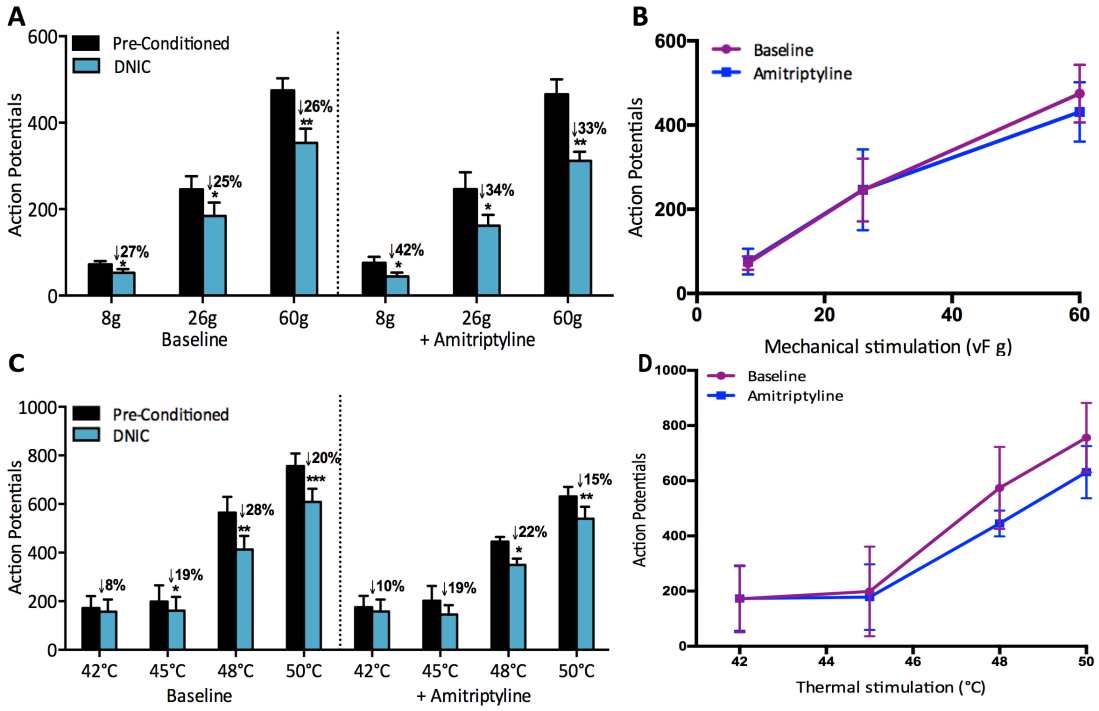


Figure 4.2. Systemic amitriptyline in naïve animals. A) The mechanically evoked neuronal responses and the degree of inhibition induced by DNIC before and after a subcutaneous injection of amitriptyline (n=6) (5mg/kg). B) The pre-conditioned mechanically evoked neuronal responses to vonFrey hairs of increasing bending force and the pre-conditioned mechanically evoked neuronal responses following Amitriptyline treatment. C) The thermally evoked neuronal responses and the magnitude of inhibition induced by DNIC before and after systemic Amitriptyline (5mg/kg) (n=6). D) The pre-conditioned thermally evoked neuronal responses to water jets of increasing temperature and the pre-conditioned thermal evoked responses following subcutaneous injection of amitriptyline. Two-way ANOVA with Bonferonni post-hoc test (*P<0.05, **P<0.01, ***P<0.001).

4.3.4 The magnitude of neuronal inhibition induced by DNIC following reboxetine, amitriptyline and fluoxetine treatment.

While the NRI reboxetine consistently increased the magnitude of inhibition induced by DNIC to both noxious mechanical and thermal stimulations, the percentage of inhibition on neuronal firing was not significantly different before drug treatment and following spinal application of reboxetine. The tricyclic antidepressant amitriptyline and the SSRI fluoxetine did not appear to affect the degree of inhibition induced by DNIC to either mechanical and thermal stimulations in naïve animals as shown in Figure 4.4.

So far, the inhibition induced by DNIC was tested in both mechanical and thermal evoked neuronal responses. At each time point 3 baseline responses were tested to obtain an average and one DNIC response was obtained, this meant the mechanical and

thermal pharmacology experiments could not be combined due to limited time for each drug time point. Due to the nature of electrophysiological recordings and to avoid accumulative doses, one animal was used per experiment. Therefore in keeping with the 3Rs of replacement, reduction and refinement (NC3Rs) the inhibition induced by DNIC was tested in mechanical evoked neuronal responses from this point on.

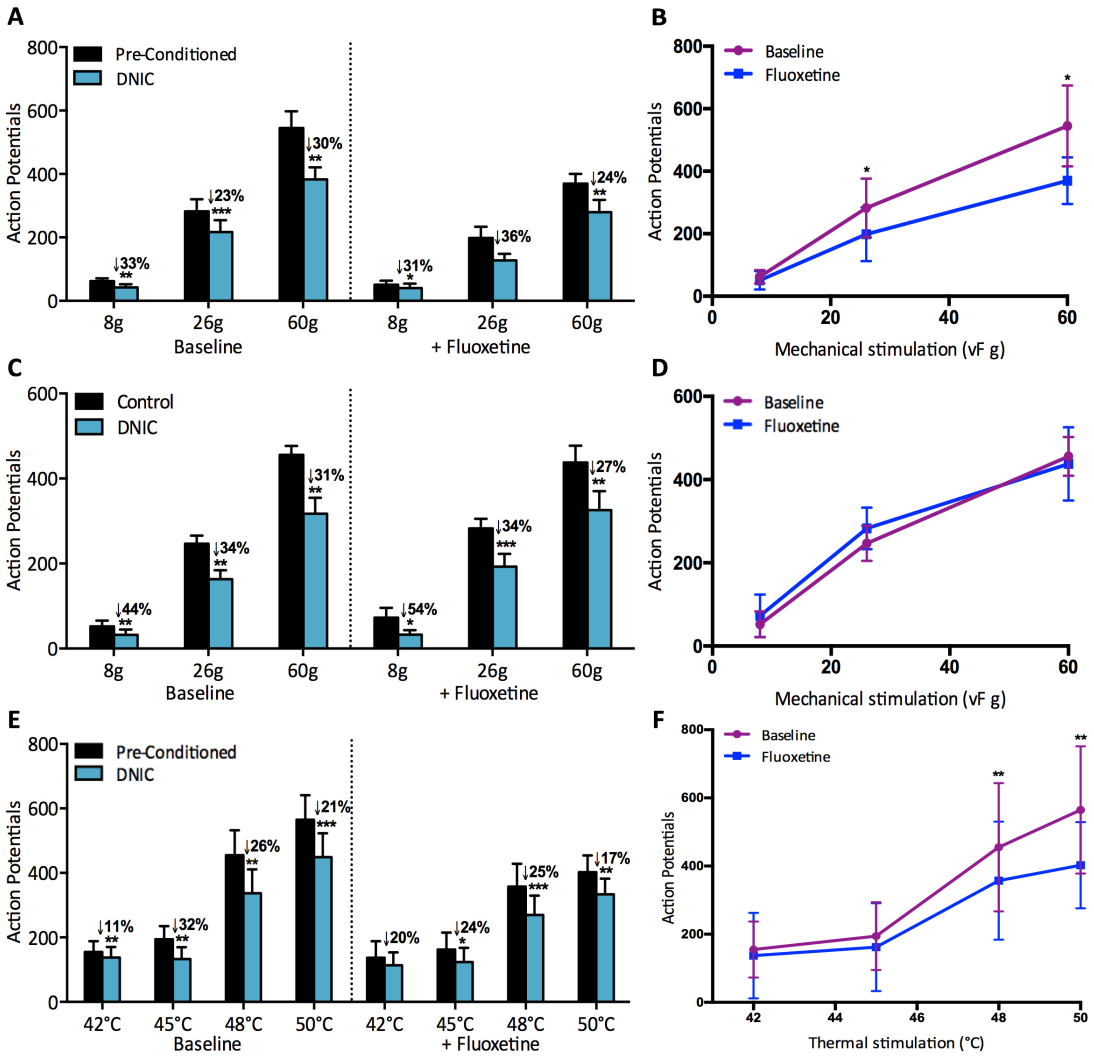


Figure 4.3 Spinal and systemic administration of fluoxetine in naïve animals. A) The mechanical evoked neuronal responses and the degree of inhibition induced by DNIC before and after spinal application of fluoxetine (100µg/50µL) (n=6). B) The pre-conditioned mechanical evoked neuronal responses were significantly reduced at 26g and 60g following spinal application of fluoxetine. C) The mechanical evoked neuronal responses and the magnitude of inhibition induced by DNIC before and after a subcutaneous injection of Fluoxetine (20mg/kg) (n=5). D) The pre-conditioned mechanical evoked neuronal responses were unaffected by the subcutaneous injection of fluoxetine. E) The thermal evoked neuronal responses and the degree of inhibition induced by DNIC before and following spinal application of fluoxetine (100µg/50µL) (n=6). F) The pre-conditioned thermal evoked neuronal responses were significantly inhibited at 48°C and 50°C. Two-way ANOVA with Bonferroni correction (*P<0.05, **P<0.01, ***P<0.001).

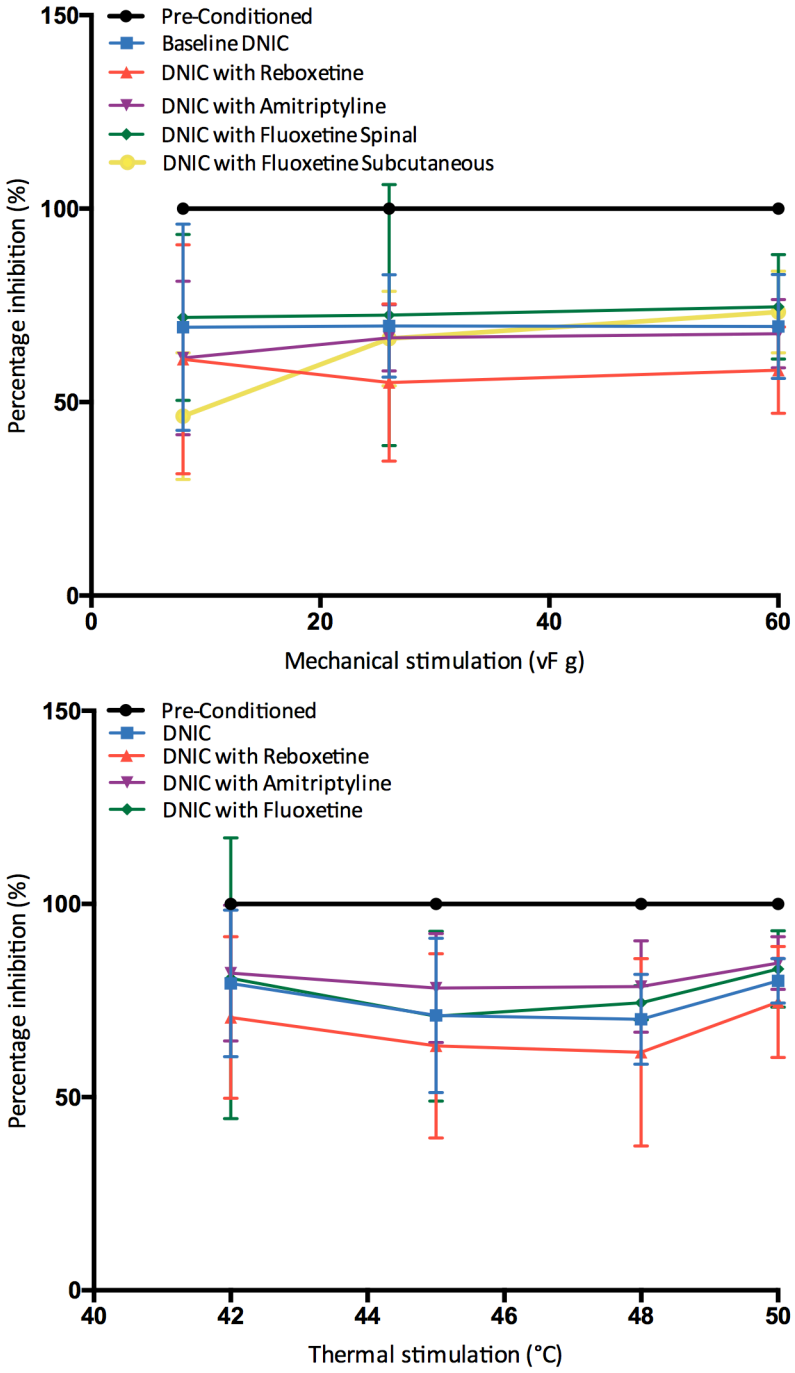


Figure 4.4. The magnitude of inhibition on neuronal firing induced by DNIC following reboxetine, amitriptyline, and fluoxetine treatment. The degree of inhibition induced by a concurrent noxious ear pinch in Naïve animals before any pharmacological treatment is compared to the degree of inhibition induced by DNIC following spinal and systemic treatment with an NRI, tricyclic antidepressant, and SSRI. A) The degree of inhibition induced by DNIC on mechanically evoked neuronal responses. This indicated that following treatment with systemic amitriptyline, systemic fluoxetine and spinal fluoxetine the degree of inhibition induced by DNIC remains similar to the levels observed pre-drug. However, the spinal application of reboxetine increases the magnitude of inhibition induced by DNIC to mechanically evoked responses, however this is not significant. B) The degree of inhibition induced by DNIC on thermally evoked neuronal responses. Following treatment with systemic amitriptyline and spinal fluoxetine the

degree of inhibition induced by DNIC remains similar to the levels observed pre-drug. Meanwhile, the spinal application of reboxetine increases the degree of inhibition induced by DNIC to thermal evoked neuronal responses, yet this effect is not significant. Kruskall-Wallis Independent Samples One Way Anova.

4.3.5 DNIC expression in SNL animals

The magnitude of neuronal inhibition induced by DNIC was then assessed in a Spinal Nerve Ligation (SNL) rat model of neuropathy. The work presented in the rest of this chapter was a collaborative study with Dr Kirsty Bannister (Bannister et al 2017). The lab has previously examined the magnitude of DNIC in Naïve and Sham operated animals and found there to be no difference in the level of neuronal inhibition upon induction of DNIC (Bannister et al 2015).

The magnitude of inhibition induced by DNIC on mechanical evoked neuronal responses was assessed in nerve ligated rats at least 14 days after surgery, the DNIC was induced by a concurrent noxious conditioning ear pinch ipsilateral to the side of injury. Interestingly and unlike naïve animals, where DNIC produced a consistent inhibition of neuronal firing of approximately 30%, no inhibition on neuronal firing was observed in SNL animals with the noxious conditioning ear pinch. The difference in DNIC responses between naïve and SNL animals is shown in Figure 4.5.

4.3.6 Fluoxetine in SNL animals

The degree of inhibition induced by DNIC on mechanically evoked neuronal firing was assessed in SNL rats following spinal and systemic administration of the SSRI fluoxetine. When the spinal content of 5-HT was increased following the spinal application of fluoxetine, mechanically evoked neuronal responses to 8g, 26g, and 60g were significantly inhibited in the presence of a concurrent noxious conditioning ear pinch by 42%, 39%, and 34% respectively (Figure 4.6a). Additionally, the spinal administration of fluoxetine significantly inhibited the pre-conditioned neuronal responses to both innocuous and noxious mechanical stimulation (Figure 4.6b). However following the subcutaneous injection of fluoxetine, there was no significant reduction in mechanically evoked neuronal firing with a concurrent noxious conditioning ear pinch (Figure 4.6c). The systemic administration of fluoxetine had no significant affect on the pre-conditioned mechanically evoked neuronal firing (Figure 4.6d). This indicates that

increased synaptic levels of 5-HT must occur specifically in the spinal cord to restore the DNIC system.

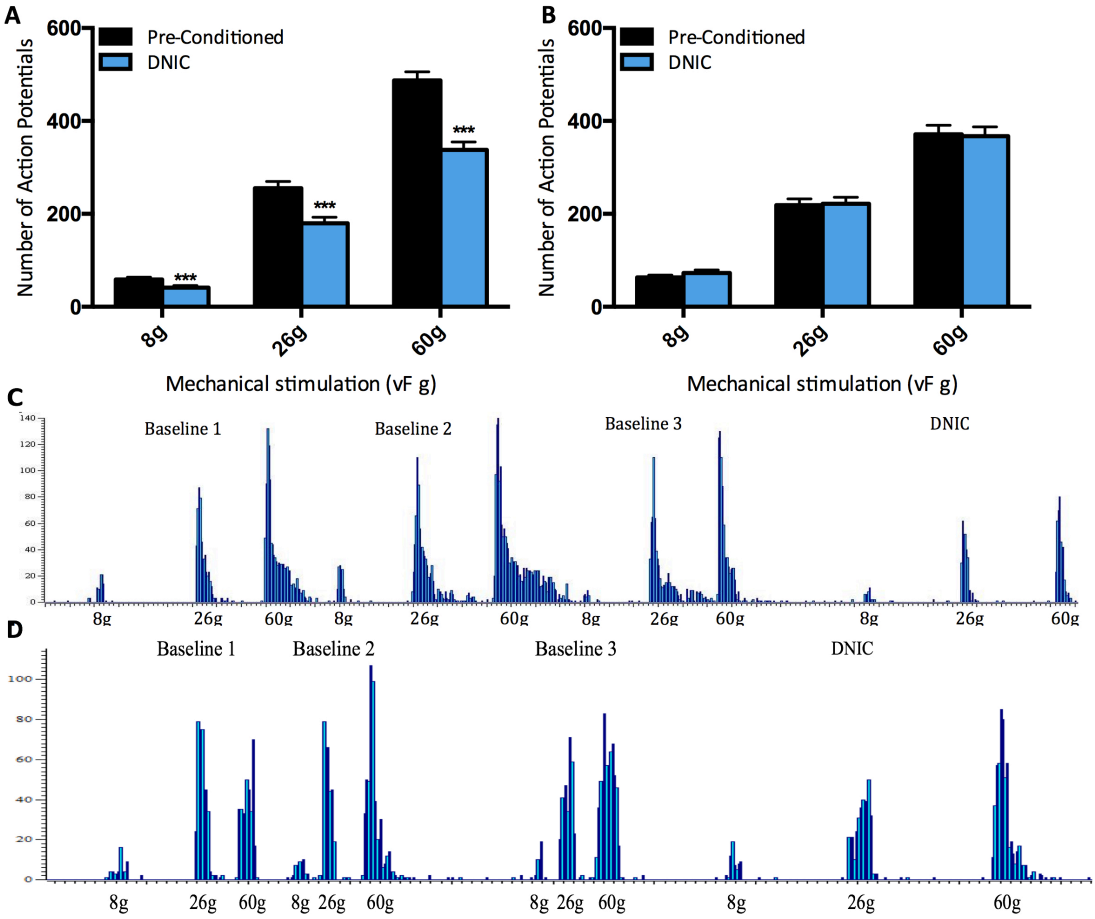


Figure 4.5 The inhibition on neuronal firing induced by DNIC in naïve and SNL rats. A) In naïve animals a concurrent noxious conditioning ear pinch produces a significant reduction in mechanically evoked neuronal firing (n=25). B) In SNL animals the concurrent noxious conditioning ear pinch does not produce any inhibition on mechanically evoked neuronal firing (n=39). C) A raw trace of one example WDR neuron from a Naïve animal; this shows three pre-conditioned neuronal responses to mechanical stimulation of vonFrey hairs of increasing bending force, upon application of a concurrent noxious conditioning ear pinch the mechanically evoked neuronal firing was inhibited. D) A raw trace of one example WDR neuron from a neuropathic animal; this demonstrates that the mechanically evoked neuronal firing is similar for both pre-conditioned responses and in the presence of a concurrent noxious conditioning ear pinch. Two-way ANOVA with Bonferroni post-hoc test (***P<0.001).

4.3.7 Citalopram in SNL animals

Citalopram is also an SSRI, which was spinally and systemically administered to neuropathic rats to further assess the effect of increased synaptic content of 5-HT on the degree of inhibition induced by DNIC. While both fluoxetine and citalopram have been demonstrated using microdialysis to increase extracellular concentration of 5-HT 2-4

fold, Fluoxetine was found to also increase the concentrations of NA and dopamine in the prefrontal cortex (Bymaster et al 2002). Citalopram was found to only cause an increase in the extracellular concentration of 5-HT and so has been suggested to be a more selective SSRI (Bymaster et al 2002). Therefore, the spinal and systemic administration of citalopram was investigated to ensure the restoration of DNIC was occurring due to increased 5-HT spinal synaptic content rather than through a NA mediated mechanism.

When the spinal content of 5-HT was increased following spinal application of citalopram, mechanically evoked neuronal responses to 8g, 26g, and 60g were inhibited with a concurrent noxious conditioning ear pinch by 22%, 46%, and 38% respectively. For the noxious mechanical stimulations of 26g and 60g the DNIC induced a significant reduction in neuronal firing (Figure 4.7a). The spinal application of citalopram did not significantly inhibit the pre-conditioned mechanically evoked neuronal firing (Figure 4.7b). Similarly to fluoxetine, the systemic administration of citalopram did not restore any degree of inhibition of neuronal firing with a concurrent noxious conditioning ear pinch (Figure 4.7c). The systemic administration of citalopram also had no effect on the pre-conditioned mechanically evoked neuronal firing (Figure 4.7d).

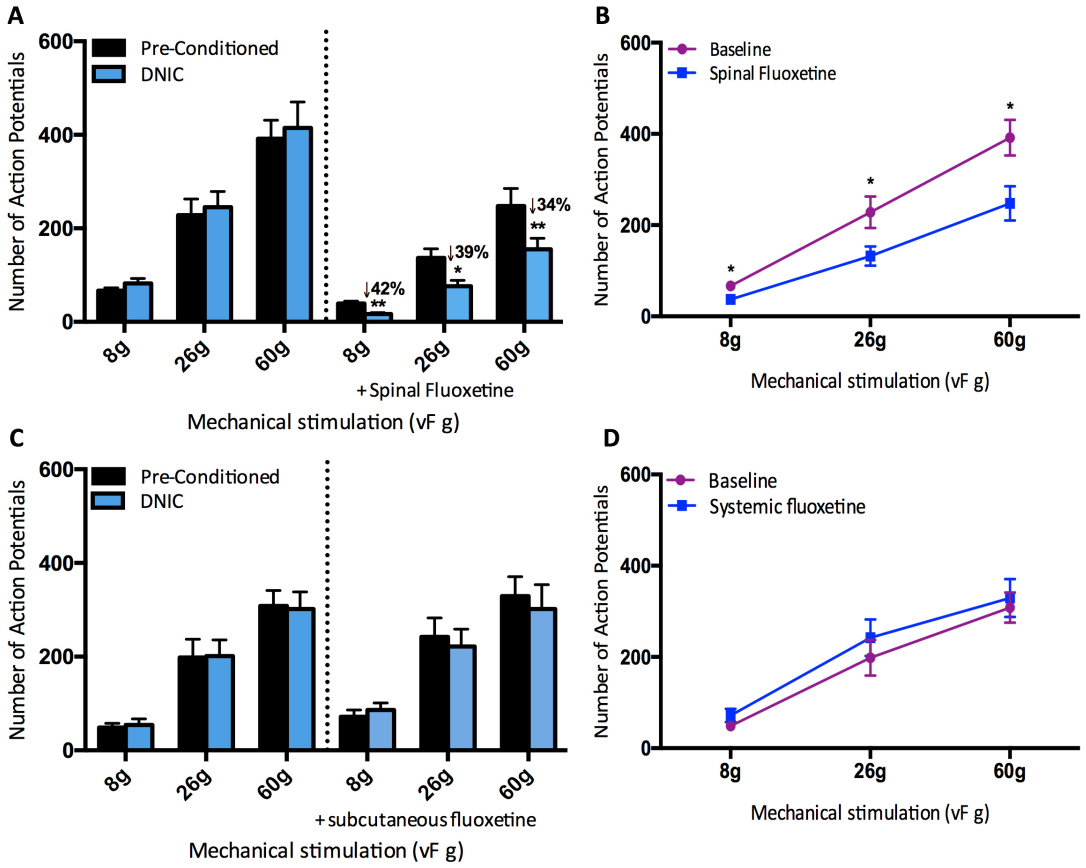


Figure 4.6 The spinal and systemic administration of fluoxetine in SNL rats. A) The mechanically evoked WDR neuronal responses and the degree of inhibition induced by DNIC following spinal application of fluoxetine (100µg/50µL) (n=6). This indicates that increasing the spinal synaptic content of 5-HT restores the DNIC system in SNL animals. B) The pre-conditioned mechanically evoked neuronal responses were significantly inhibited following the spinal application of fluoxetine (100µg/50µL) (n=6). C) The pre-conditioned mechanically evoked neuronal responses and the neuronal responses with a concurrent noxious conditioning ear pinch, subcutaneous injection of fluoxetine (20mg/kg) did not restore the DNIC system in SNL animals (n=5). D) The pre-conditioned mechanically evoked neuronal responses were not significantly affected by a systemic injection of fluoxetine (20mg/kg) (n=5). Two-way ANOVA with Bonferroni post-hoc test (*P<0.05, **P<0.01).

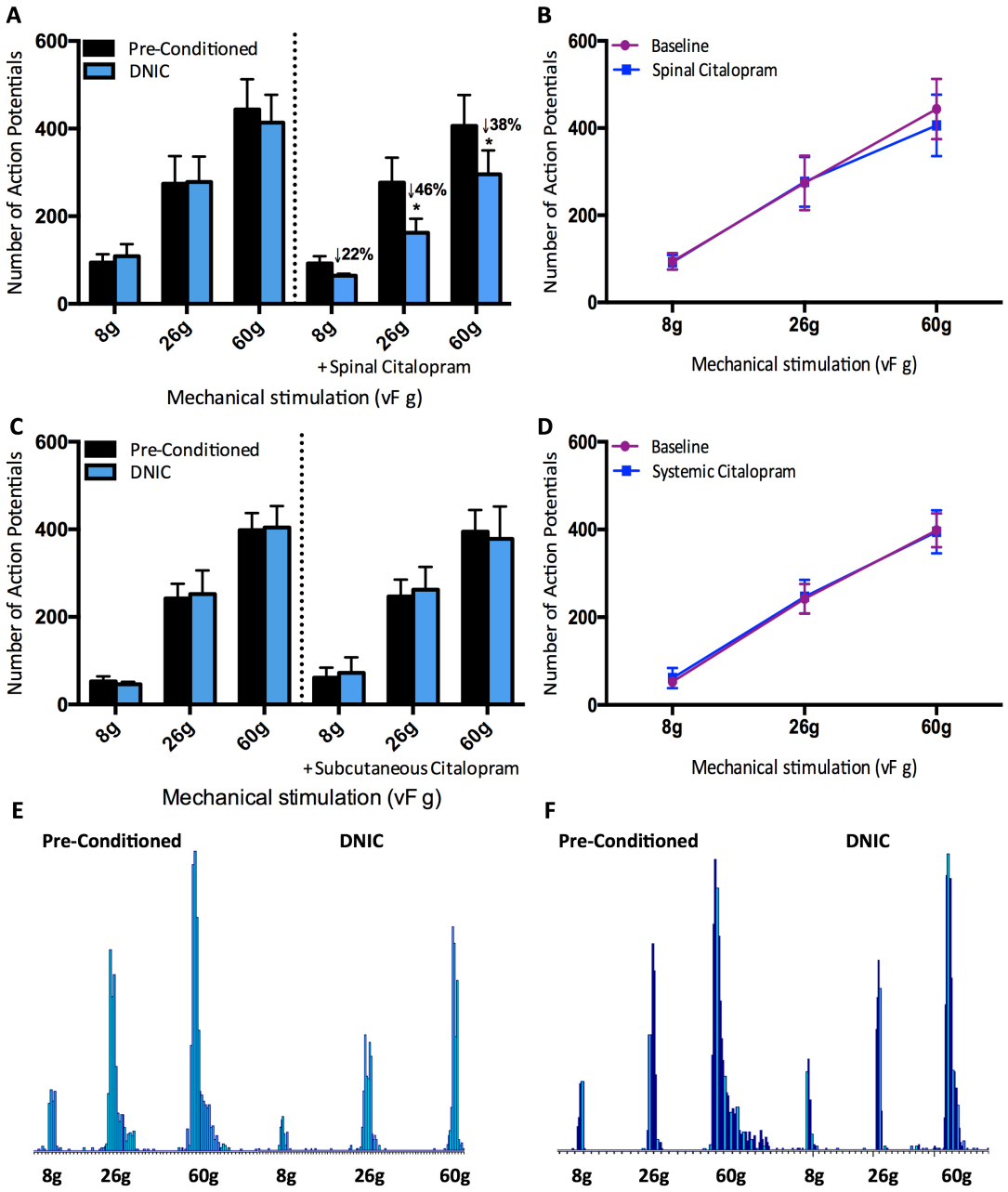


Figure 4.7. The spinal and systemic administration of citalopram in SNL animals. A) The mechanically evoked neuronal responses and the magnitude of inhibition induced by DNIC before and after spinal application of citalopram (100µg/50µL) (n=6). The spinal application of citalopram results in a significant reduction in mechanically evoked neuronal firing with the concurrent noxious conditioning ear pinch. B) The spinal citalopram does not significantly inhibit the pre-conditioned mechanically evoked neuronal responses. C) A subcutaneous injection of citalopram (10mg/kg) does not produce a significant reduction of mechanically evoked neuronal firing with a concurrent noxious conditioning ear pinch (n=5). D) Systemic Citalopram does not affect the pre-conditioned mechanically evoked neuronal responses. E) A raw trace of one example WDR neuron from an SNL rat following spinal application of citalopram. One example pre-conditioned response is shown and the DNIC response shows there is a reduction in mechanically evoked neuronal firing in the presence of the concurrent noxious conditioning ear pinch. F) A raw trace from one example WDR neurons from an SNL rat following subcutaneous injection of citalopram. This trace shows that there is no reduction in mechanically evoked neuronal firing with a concurrent noxious conditioning ear pinch. Two-Way ANOVA with Bonferroni post-hoc test (*P<0.05).

4.3.8 Co-administration of spinal selective serotonin reuptake inhibitors and a 5-HT_7 receptor antagonist

The increased spinal synaptic content of 5-HT restored DNIC in SNL animals, therefore the inhibitory effect must be caused by 5-HT receptor mediated actions. As the 5-HT_7 receptor has recently been reported to play an antinociceptive role at the level of the spinal cord, a selective 5-HT_7 receptor antagonist SB-267790 was co-administered to the spinal cord along with Fluoxetine or Citalopram. Interestingly, when SB-267790 was applied to the spinal cord with Fluoxetine or Citalopram the inhibitory effect was blocked. There was no longer a reduction of mechanically evoked neuronal firing in the presence of a concurrent noxious conditioning ear pinch, indicating a complete block on the restoration of DNIC. This indicates that increased 5-HT content in the spinal cord can activate 5-HT_7 receptors to mediate an inhibition of the pain response.

4.3.9 Co-administration of spinal selective serotonin reuptake inhibitors and an α_2-adrenergic receptor antagonist

DNIC has been shown to rely on a NA mechanism acting through α_2-adrenergic receptors in the spinal cord (Bannister et al 2015). Therefore the degree of inhibition on neuronal firing following spinal application of Fluoxetine and Citalopram was analysed when these SSRIs were co-administered spinally with an α_2-adrenergic receptor antagonist Atipamezole. Remarkably Atipamezole blocked the reduction in mechanically evoked neuronal firing with a concurrent noxious conditioning ear pinch as shown in Figure 4.9. This indicates that even with increased extracellular levels of 5-HT in the spinal cord, NA actions through the α_2-adrenergic receptors are required for DNIC to function.

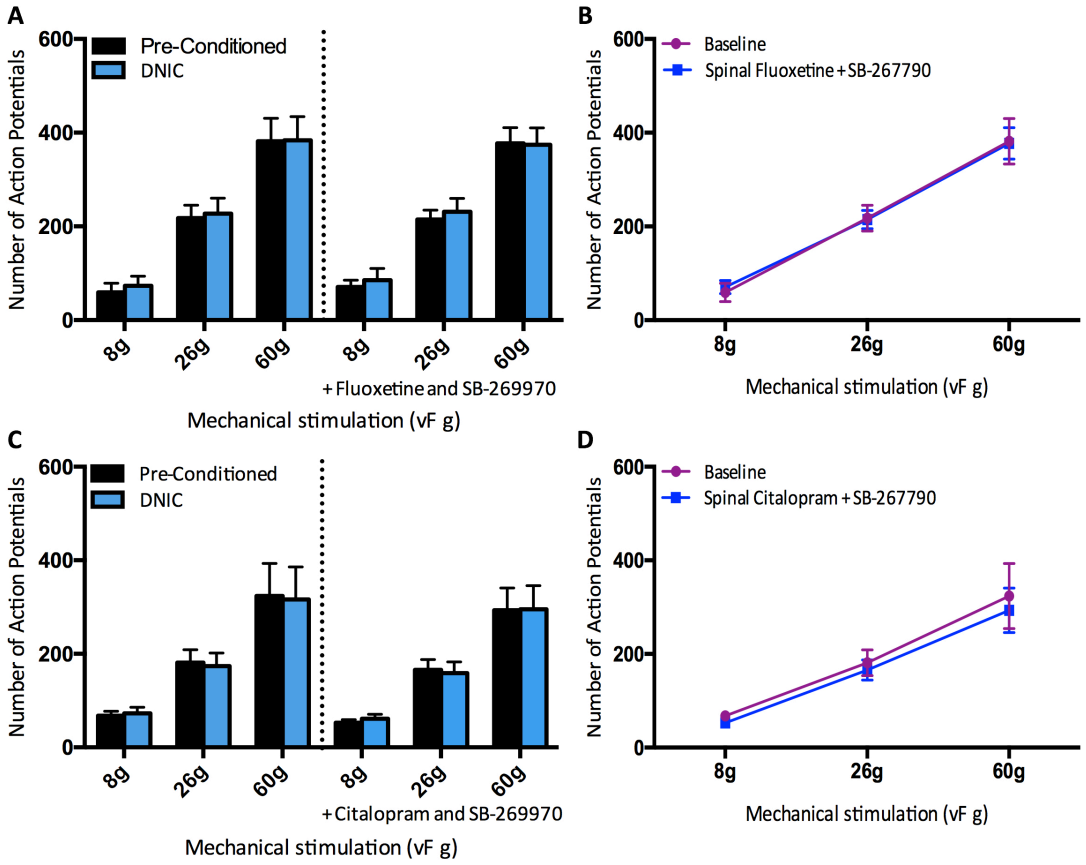


Figure 4.8. The co-administration of fluoxetine or citalopram with the 5-HT$_7$ antagonist SB-267790 in SNL rats. A) The mechanically evoked neuronal responses and the degree of inhibition induced by a concurrent noxious conditioning ear pinch in SNL animals before and after the spinal co-administration of fluoxetine (100µg/50µL) and SB-267790 (100µg/50µL) (n=5). The 5-HT$_7$ antagonist blocked the inhibitory actions and restoration of DNIC observed with fluoxetine alone. B) The spinal co-administration had no affect on the pre-conditioned mechanically evoked neuronal responses. C) The mechanically evoked neuronal responses and the degree of inhibition induced by a concurrent noxious conditioning ear pinch in SNL rats before and after spinal application of citalopram (100µg/50µL) and SB-267790 (100µg/50µL). There is no reduction in mechanically evoked neuronal firing with a concurrent noxious ear pinch following citalopram and SB-267790 indicated SB-267790 blocks the inhibitory actions of citalopram applied alone. D) The pre-conditioned mechanically evoked neuronal responses were unaffected by the spinal co-administration of citalopram and SB-267790. Two-way ANOVA with Bonferonni post-hoc test.

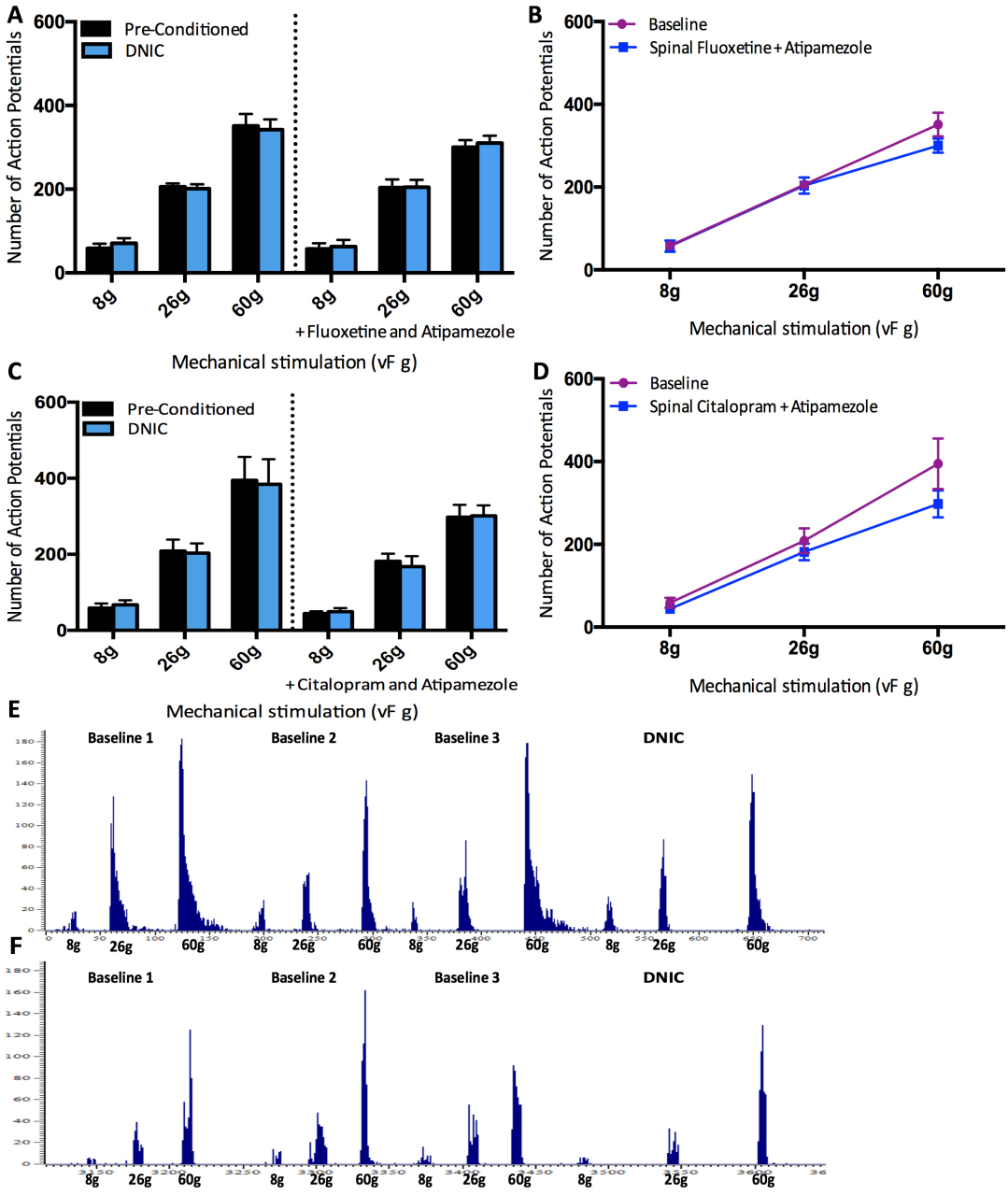


Figure 4.9 The co-administration of fluoxetine or citalopram with the α_2-adrenergic receptor atipamezole. A) The mechanically evoked WDR neuronal responses and the degree of inhibition induced by a concurrent noxious conditioning ear pinch in SNL rats before and after the spinal co-administration of fluoxetine (100µg/50µL) and atipamezole (100µg/50µL) (n=6). Atipamezole blocks the inhibitory action of fluoxetine as there is no reduction of neuronal firing induced by DNIC as is seen when fluoxetine is administered alone. B) The pre-conditioned mechanically evoked neuronal responses were not significantly affected by the co-administration of fluoxetine and atipamezole. C) The mechanically evoked WDR neuronal responses and the magnitude of inhibition induced by a concurrent noxious conditioning ear pinch in SNL animals before and after the spinal co-administration of citalopram (100µg/50µL) and atipamezole (100µg/50µL) (n=6). The spinal co-administration of citalopram and atipamezole did not produce a reduction in mechanically evoked neuronal firing with a concurrent noxious conditioning ear pinch. D) The pre-conditioned mechanically evoked neuronal responses were reduced following spinal co-administration of Citalopram and Atipamezole but this was not significant. E) A raw trace from one WDR neuron in an SNL rat showing baseline responses before drug treatment. This shows three pre-conditioned mechanically evoked neuronal responses and mechanically evoked neuronal responses in the presence of a concurrent noxious

conditioning ear pinch. F) A raw trace from the WDR neuron in an SNL rat following spinal co-administration of Citalopram and Atipamezole. The trace shows three pre-conditioned mechanically evoked neuronal responses and the mechanical evoked responses in the presence of a noxious conditioning ear pinch. The spinal co-administration of Citalopram and Atipamezole does not reduce the mechanically evoked neuronal responses in the presence of a noxious conditioning ear pinch. Two-way ANOVA with Bonferroni post-hoc test.

4.4 Discussion

4.4.1 The balance in descending noradrenergic and serotonergic controls in naïve animals

This study provides further evidence that a concurrent noxious conditioning stimulus reliably and consistently inhibits the evoked activity of convergent WDR neurons in naïve rats. The DNIC system has previously been reported to rely on NA mechanisms acting through α_2-adrenergic receptors as blockade of this receptor abolished DNIC in naïve animals (Bannister et al 2015). Descending modulatory systems arising in the brainstem can exert bi-directional controls on pain perception at the level of the spinal cord through the release of monoamines (Bannister and Dickenson 2016b). Under normal conditions the concurrent noxious conditioning stimulus is thought to activate descending inhibitory pathways to prompt NA release in the dorsal horn, which can activate α_2-adrenergic receptors to mediate inhibition of the pain response. This study explored the roles played by both NA and 5-HT in mediating DNIC further by increasing the synaptic availability of NA and 5-HT using an NRI, SSRI, and tricyclic antidepressant, and investigating the subsequent effects on the magnitude of inhibition induced by DNIC.

4.4.1.1 Reboxetine in Naïve animals

This study demonstrates that spinal application of the NRI reboxetine increases the degree of inhibition induced by DNIC in naïve animals. This demonstrates that increasing the synaptic content of NA amplifies the endogenous inhibitory system utilized by DNIC in Naïve animals. This suggests that in naïve animals the application of the noxious conditioning ear pinch activates a descending noradrenergic inhibitory system, which dominates over the descending facilitatory system to trigger DNIC and cause a reduction in WDR neuronal firing.

The importance of the NA system in mediating DNIC has previously been described in both animal models and human subjects with chronic pain. The spinal application of reboxetine has also been demonstrated to restore DNIC in a spinal nerve ligation rat model of neuropathy (Bannister et al 2015). Furthermore in patients with diabetic polyneuropathy and a dysfunctional CPM system, tapentadol (a μ-opioid receptor agonist and NRI) treatment activated the CPM system (Niester et al 2014). Interestingly, intrathecal reboxetine was found to partially reverse mechanical allodynia in rats with

tibial nerve transection, which was blocked by intrathecal administration of the α_2-adrenoceptor antagonist yohimbine. However, the α_1-adrenoceptor antagonist prazosin and the β-adrenoceptor antagonist propranolol had no effect on the antinociceptive actions of reboxetine (Hughes et al 2015). These findings indicate that the endogenous inhibitory system utilized by DNIC can be restored through pharmacologically modulating noradrenergic descending inhibitory controls by increasing the synaptic levels of NA, which can ultimately activate inhibitory α_2-adrenoceptors. Restoring DNIC by enhancing the noradrenergic inhibitory systems indicates that the loss of DNIC in chronic pain states is likely due to reduced activity of the noradrenergic descending inhibitory controls arising in the brainstem.

The importance of a functional endogenous inhibitory system utilized by DNIC and the noradrenergic inhibitory descending controls that sub-serve this system have been further demonstrated in the clinic. Firstly a correlation has been demonstrated between pain-free patients with an inefficient CPM and the development of chronic pain following thoracotomy surgery (Yarnitsky et al 2008). This indicates that patients with an inefficient endogenous analgesic system are more likely to develop chronic pain in the future (Yarnitsky et al 2008). Secondly in patients with painful diabetic neuropathy, the efficiency of the SNRI duloxetine was found to be correlated with CPM efficiency, such that patients with an inefficient CPM would benefit most from the drug (Yarnitsky et al 2012). This demonstrates that increasing the synaptic availability of NA in patients with inefficient CPM may be sufficient to restore the endogenous inhibitory system mediated by DNIC, and reduce their likelihood of developing chronic pain in the future.

Overall, the ability of increased extracellular levels of NA to amplify the inhibition induced by DNIC in naïve animals confirms the importance of NA actions at spinal α_2-adrenoceptors in mediating DNIC. Furthermore, while reboxetine increased the degree of inhibition induced by DNIC, amitriptyline and fluoxetine treatment that act to increase the synaptic availability of both NA and 5-HT or specifically 5-HT, do not produce this effect. This indicates that NA may be the monoamine that holds the greatest potential for mediating the inhibition induced by DNIC.

4.4.1.2 Amitriptyline in Naïve animals

The effect of the tricyclic antidepressant, amitriptyline, on WDR neuronal firing and magnitude of inhibition induced in DNIC was then investigated in naïve animals.

Amitriptyline is primarily a serotonin-noradrenaline reuptake-inhibitor (SNRI) but has a less specific mode of action than reboxetine and fluoxetine, it also blocks histamine H_1, muscarinic, and NMDA receptors giving it a sedative and hypotensive effect too (Bomholtet al 2005, Sindrup et al 2005). Tricyclic antidepressants are the most widely used antidepressant drugs in the treatment of neuropathic pain, they were found to be more effective than SNRIs and SSRIs at relieving peripheral neuropathic pain in terms of numbers needed to treat (Sindrup et al 2005). Interestingly in this study, the subcutaneous injection of Amitriptyline had little effect on pre-conditioned mechanical and thermal evoked neuronal responses and on the degree of inhibition induced by DNIC.

As amitriptyline functions primarily as a serotonin and noradrenaline reuptake inhibitor, the effect on the degree of inhibition induced by DNIC was tested with the increased synaptic availability of 5-HT and NA. The subcutaneous injection of amitriptyline had little effect on the magnitude of inhibition induced by DNIC, with the inhibition on mechanical and thermal evoked neuronal firing upon application of the noxious ear pinch at similar levels before and after drug administration. Although amitriptyline has been demonstrated to be an effective analgesic in both neuropathic and inflammatory pain states, the exact analgesic mechanisms have not been conclusively determined (Acton et al 1992, Sawynok et al 1999, Sindrup et al 2005) Amitriptyline has been shown to have an effective analgesic effect in patients with tension-type headache (Ashina et al 2004). To better understand the analgesic mechanisms in tension-type headache the authors compared Amitriptyline's analgesic effect and efficiency to prevent 5-HT presynaptic reuptake to the SSRI Citalopram by measuring platelet 5-HT levels in blood samples from treated patients (Ashina et al 2004). The authors found that while amitriptyline was much more effective in relieving the pain associated with the headaches, citalopram was much more effective in inhibiting the reuptake of 5-HT, indicating amitriptyline's analgesic effects are likely due to other mechanisms of action (Ashina et al 2004). Amitriptyline being relatively ineffective at preventing the presynaptic reuptake of 5-HT and NA may explain why it is does not enhance the magnitude of inhibition induced by DNIC in this study.

The subcutaneous injection of amitriptyline also had little effect on the pre-conditioned mechanically and thermally evoked WDR neuronal responses, amitriptyline did reduce neuronal firing in response to the more noxious thermal stimulations of 48°C and 50°C but this effect was not significant. Similarly, previous findings have reported that

amitriptyline is more effective at relieving thermally evoked pain than mechanically evoked pain. In a rat ligation model of neuropathy, amitriptyline reversed thermal hyperalgesia yet had no effect in alleviating mechanical allodynia (Esser and Sawynok 1999). Similarly, in rats with chronic constriction injury amitriptyline was demonstrated to fully reverse thermal hypersensitivity yet could only slightly reduced mechanical hyperalgesia and could not attenuate mechanical allodynia (Bomholt et al 2005). However, the authors also found that amitriptyline was ineffective at modulating the nocicieptive response latencies in naïve rats during the hot plate test and was also ineffective at relieving the aversive behavior observed with the formalin test (Bomholt et al 2005). These findings suggest that the analgesic properties of amitriptyline are inconsistent and may depend upon the chronic pain model, but our electrophysiological results suggest that amitriptyline has a limited effect on neuronal responses in naïve animals.

Finally, amitriptyline was delivered by a subcutaneous injection rather than the spinal administration used for reboxetine and fluoxetine, this may account for the ineffectiveness of amitriptyline on neuronal firing. This route was chosen due to previous experiments in the lab that found spinal administration of amitriptyline caused neuronal damage and prevented electrophysiological recordings. However in a spinal nerve ligation rat model of neuropathy, amitriptyline was found to have an antihyperalgesic effect when administered through intrathecal, intraperitoneal, and subcutaneous injection (Esser and Sawynok 1999). A subcutaneous injection also most accurately represents the systemic administration of amitryptiline as would be the case in the clinic.

Overall while Amitriptyline has been demonstrated to have analgesic properties in models of chronic pain, it may not be the best pharmacological tool for investigating the function of NA and 5-HT in mediating DNIC.

4.4.1.3 Fluoxetine in Naïve animals

The effect of a spinal application of the SSRI fluoxetine on mechanically and thermally evoked WDR neuronal firing and the degree of inhibition induced by DNIC was investigated. Interestingly the spinal application of fluoxetine significantly inhibited the pre-conditioned noxious mechanical and thermal evoked neuronal firing, suggesting when the 5-HT content in the cord is increased an overriding inhibitory pathway

dominates. However, the spinal application of fluoxetine had little effect on the magnitude of inhibition induced by DNIC, indicating that increased extracellular 5-HT cannot enhance the degree of inhibition on WDR neuronal firing upon concurrent application of the noxious conditioning ear pinch.

This study suggests that when the SSRI fluoxetine increases the spinal synaptic levels of 5-HT, 5-HT mediates an inhibitory action. The role played by 5-HT in nociception is complex due to myriad of 5-HT receptors and alterations in the serotonergic system in chronic pain states, therefore studies have shown conflicting findings as to whether the serotonergic descending faciliatory or inhibitory pathways originating in the RVM predominate. Recently, Cai et al demonstrated that optogenetic stimulation of 5-HT neurons in the RVM decreased both mechanical and thermal pain thresholds, suggesting 5-HT exerts a pre-dominant facilitatory effect under normal conditions (Cai et al 2014). A contrasting study investigated spinally projecting serotonergic neurons arising in the RVM, they found these neurons expressed the Neurotensin Receptor Subtype 1 (NRT1), and found an agonist for this receptor produced dose-dependent antinociception in the tail-flick test (Buhler et al 2005). The authors suggested that NRT1-expressing serotonergic neurons arising in the RVM that project to the spinal cord mediate antinocieption through the release of 5-HT in the dorsal horn (Buhler et al 2005). In addition, intrathecal administration of the selective 5-HT enhancer Tianeptine was demonstrated to dose-dependently attenuate mechanical allodynia in a spinal nerve ligation rat model of neuropathy (Lin et al 2015). Therefore, the facilitatory or inhibitory actions mediated by 5-HT may be determined by the receptor activated, the pain state, and 5-HT levels present.

Unlike the inhibitory actions mediated by spinal application of fluoxetine, a subcutaneous injection of fluoxetine had little effect on mechanically evoked WDR neuronal responses or the degree of inhibition induced by DNIC. This indicates that increased synaptic levels of 5-HT need to occur specifically in the spinal cord to have an inhibitory effect. Although fluoxetine crosses the blood-brain-barrier (Warren 2012), the systemic administration of fluoxetine may not be sufficient to raise 5-HT synaptic levels enough in the spinal cord to mediate inhibitory actions. In addition, studies have demonstrated that peripheral and central 5-HT receptors play opposing roles in nociception. In both inflammatory and neuropathic conditions, a peripherally applied 5-HT_7 receptor agonist produced pronociceptive effects while the central application of the 5-HT_7 receptor agonist produced antinociceptive effects (Brenchat et al 2012).

These findings may suggest that the subcutaneous injection of fluoxetine produces opposing centrally and peripherally mediated effects, which may balance out and prevent the increased 5-HT synaptic content from mediating an inhibition on neuronal firing.

4.4.2 The actions of serotonin in SNL animals

This study confirmed that DNIC is abolished in an SNL rat model of neuropathy, which is in agreement with previous findings (Bannister et al 2015). Previous studies have demonstrated that DNIC can be restored in SNL animals with the NRIs, Reboxetine and Tapentadol, and also by blocking the actions of 5-HT_3 receptor with the antagonist Ondansetron (Bannister et al 2015). These findings suggest that following neuropathy neurobiological mechanisms cause an inactivation of DNIC, whereby the noradrenergic descending inhibitory system becomes quiescent and the serotonergic facilitatory descending inhibitory system acting through 5-HT_3 receptors becomes upregulated. The balance that exists between descending facilitation and inhibition is abolished such that the activation of inhibitory controls upon application of the noxious conditioning stimulus is no longer sufficient to override facilitation. This study investigated the role of 5-HT at the level of the spinal cord further in SNL animals.

4.4.2.1 Selective Serotonin Re-uptake inhibitors in SNL animals

The spinal application of the SSRIs Fluoxetine and Citalopram restored DNIC in SNL animals, the mechanically evoked neuronal firing was now significantly inhibited upon application of a concurrent conditioning noxious ear pinch. This suggests that when the synaptic levels of 5-HT are increased in the spinal cord, 5-HT can predominantly mediate inhibitory actions. This was interesting as the serotonergic facilitatory system acting at the 5-HT_3 receptor is known to be enhanced following neuropathy, however it appears when the extracellular 5-HT levels are increased further in the dorsal horn 5-HT can predominantly mediate inhibitory actions (Suzuki et al 2002, Dogrul et al 2009). Previous studies have demonstrated that descending serotonergic pathways are required for the full inhibitory effects of DNIC (Dickenson et al 1981, Lindstedt et al 2011). Dickenson et al found that depletion of 5-HT in the spinal cord strongly reduced the magnitude of inhibition induced by DNIC (Dickenson et al 1981). In addition, patients with low expression of the 5-HT transporter have a less efficient CPM (Linstedt

et al 2011). Overall the pronociceptive or antinociceptive direction of 5-HT in the spinal cord appears to depend upon the 5-HT levels and subsequent receptor activated.

Interestingly, the subcutaneous injection of the SSRIs Fluoxetine and Citalopram did not restore the neuronal inhibition induced by DNIC in SNL animals. This is similar to the findings in Naïve animals where spinal, but not systemic, Fluoxetine inhibited mechanically evoked neuronal firing. This suggests that for the increased synaptic availability of 5-HT to mediate an inhibitory action and predominate over the 5-HT_3 receptor mediated facilitation, the increased levels of 5-HT must exist specifically in the spinal cord. Both Fluoxetine and Citalopram cross the blood brain barrier but they may not increase levels of 5-HT in the spinal cord a sufficient amount to mediate inhibition (Warren 2012, Paulzen et al 2016). Furthermore, there may be opposing nociceptive actions mediated by activation of peripheral and central 5-HT receptors (Brenchat et al 2012).

4.4.2.2 The inhibitory actions of Selective Serotonin reuptake inhibitors in the spinal cord are mediated by the 5-HT_7 receptor

Remarkably, the 5-HT_7 receptor antagonist SB-267790 completely blocked the inhibitory actions mediated by spinally administered Fluoxetine and Citalopram. This would suggest that when the spinal synaptic levels of 5-HT are increased further, 5-HT can now mediate a 5-HT7 receptor mediated inhibition, that can overcome the 5-HT_3 receptor mediated facilitation. This is in agreement with previous findings that suggest an antinociceptive role for the 5-HT_7 receptor at the level of the spinal cord (Dogrul et al 2006, Dogrul et al 2009). Both a subcutaneous injection and RVM microinjection of morphine produce antinociception, in both cases the antinociceptive effects were blocked with spinal application of the 5-HT_7 receptor antagonist SB-267790 (Dogrul and Seyrek 2006, Dogrul et al 2009). This suggests that in addition to morphine's activation of opioid receptors in the brain and spinal cord, it activates serotonergic neurons in the RVM to release 5-HT in the spinal cord and mediate antinociception through 5-HT_7 receptors. As DNIC has also been shown to rely partly on opiods, it may be that combinations of these systems arising in the RVM are required for the expression of DNIC (Le Bars et al 1981). Additionally, a 5-HT_7 receptor agonist was found to induce c-Fos expression in the nucleus tractus solitaris and Parabrachial area in the brainstem, suggesting supraspinal mechanisms might also be involved in 5-HT_7 receptor mediated antinociception (Viguier et al 2012). This further indicates that the 5-HT_7 receptor is likely to be involved in the spinal inhibition induced by a noxious conditioning stimulus

as DNIC is not present in animals with spinal cord transection (Le Bars et al 1979b, Dickenson and Le Bars 1983).

In addition, the 5-HT_7 receptors in the dorsal horn have found to be localized to opiodergic and GABAergic interneurons suggesting activation of 5-HT_7 may mediate inhibition at the level of the spinal cord through the activation of inhibitory interneurons (Brenchat et al 2010, Viguier et al 2012). Interestingly in mice with partial sciatic nerve ligation there was an increase in 5-HT_7 receptor density in the ipsilateral dorsal horn, suggesting there may be a neuropathy induced up-regulation of the 5-HT_7 receptor (Brenchat et al 2010). Therefore, an increase in the synaptic level of 5-HT and increase in the 5-HT_7 receptors available for activation may be responsible for mediating inhibition at the level of the spinal cord.

4.4.2.3 The actions of spinal noradrenaline at α_2-adrenergic receptors in SNL animals

Curiously, the spinal application of an α_2-adrenergic receptor antagonist Atipamezole also blocked the inhibitory actions of spinal application of Fluoxetine and Citalopram in SNL animals. As has been previously demonstrated in both naïve and neuropathic animals, the NA mediated actions through the α_2-adrenergic receptor are vital for the expression of DNIC (Bannister et al 2015). The noradrenergic descending controls that mediate inhibition at the level of the spinal cord through activation of α_2-adrenergic receptors has been found to be downregulated following neuropathy (Rahman et al 2008). However these findings would suggest that there is still a background inhibitory noradrenergic tone, albeit at much lower levels than in the naïve state. Therefore, it may be that this inhibitory noradrenergic system must be present even at low levels for DNIC to be induced.

4.4.3 Concluding remarks

This study demonstrates that DNIC is a complex endogenous inhibitory system that relies on both noradrenergic and serotonergic descending inhibitory systems to mediate a reduction in convergent neuronal firing. However, the experiments in naïve animals demonstrate that NA may be the more important neurotransmitter for the expression of DNIC as increasing the synaptic availability of NA with Reboxteine enhances the degree of inhibition induced by DNIC, whereas increasing the synaptic availability of 5-HT with Fluoxetine does not have this effect. However when DNIC is lost in a model of

neuropathy, increasing the synaptic levels of 5-HT in the spinal cord with Fluoxetine and Citalopram restored this endogenous inhibitory system. This study proposes a pharmacological mechanism for this inhibition as the 5-HT_7 antagonist SB-267790 completely blocks the inhibition on mechanically evoked neuronal firing upon application on the noxious conditioning ear pinch. Serotonergic facilitatory descending controls mediating pronociception at the level of the spinal cord through activation of 5-HT_3 receptors are thought the be enhanced following neuropathy (Green et al 2000, Suzuki et al 2002). However this study demonstrates that when the spinal 5-HT content is raised further, 5-HT can now switch to mediate an antinociceptive role through the activation of 5-HT_7 receptors located on inhibitory interneurons. Furthermore, even low levels of noradrenergic neurotransmission acting at α_2-adrenergic receptors in the dorsal horn must occur to produce an inhibition of neuronal firing, as abolishment of this system with Atipamezole completely blocks the inhibitory actions and restoration of DNIC, even with raised synaptic levels of 5-HT following spinal administration of Fluoxetine and Citalopram. This proposes that while a low-level tonic noradrenergic inhibitory descending system must be present for DNIC to induce an inhibition on WDR neuronal firing, the conditioning noxious stimulation can evoke further noradrenergic and serotonergic descending inhibitory controls.

5. Investigating the expression of Diffuse Noxious Inhibitory Controls in a monoiodoacetate model of Osteoarthritis.

5.1 Introduction

5.1.1 Modeling Osteoarthritis

Animal models of OA provide powerful tools to investigate the mechanisms behind how OA arises and progresses. Understanding the structural pathology that occurs in the knee and the mechanisms involved in the development of chronic pain using animals that accurately model OA can help with the pre-clinical development of new therapies (Little and Zaki 2012). Multiple animal models of OA have been designed that show varied histological, behavioural and biochemical changes. The OA-like structural joint damage and associated pain can be initiated by different methods, but mostly OA is surgically, chemically, and genetically induced (Bendele 2001).

The surgically induced OA models work on the principal of joint instability and involve transection of the medial meniscus, medial collateral ligament, or anterior cruciate ligament (Pond and Nuki 1973, Janusz et al 2002, Hayami et al 2006). Most surgical models focus on the knee joints, where a loss of cartilage is often observed in the unstable knees (Brandt et al 1991). These surgically induced models have been demonstrated to mimic both nociceptive and neuropathic OA-like pain, and behaviourally animals display changes in weight bearing and develop tactile allodynia (Bove et al 2006). The surgically induced models also show a similar histopathological progression to human OA, with an initial cartilage surface, proteoglycan and chondrocyte loss, followed by vascular invasion and osteophyte formation (Junusz et al 2002, Hayami et al 2006). In the anterior cruciate ligament transection (ACLT) model the ligament is destroyed and produces a more severe disease, with extensive subchondral bone erosion and so may not be the most accurate means for modeling a slowly progressing OA phenotype (Glasson et al 2007, Lorenz and Grassel 2014).

The use of transgenic and knockout mice to model OA have provided information on the molecular mechanisms that may induce cartilage degradation and the potential genetic defects that may predispose individuals to develop OA (Goldring 1998). Transgenic mice models often involve genetic modifications affecting cartilage matrix components.

Transgenic mice with deletions in the type II procollagen gene develop erosions of the articular cartilage followed by bony sclerosis, degeneration of the meniscus and exposure of the subchondral bone, which is similar to the phenotype observed in human OA suggesting this model may be a useful tool for understanding the mechanisms involved in initiation of disease (Helminen et al 1993, Saamanen et al 2000). Similarly, transgenic mice over-expressing the matrix metalloproteinase MMP-13 display joint pathology similar to that in OA, including damage to the articular cartilage and proteoglycan loss (Neuhold et al 2001). However, mutating genes required for the expression of crucial ECM components can cause other symptoms in the mice not related to OA such as a generally disturbed growth (Helminen et al 1993).

There are also naturally occurring animal models of OA where disease pathogenesis develops in joints over time; this has been demonstrated in aging guinea pigs, mice and non-human primates (Nordling et al 1992, Carlson et al 1996, Jimenez et al 1997). Histological changes similar to human OA can be observed in the joints of these animals as they age and these changes become more severe as the animals age (Carlson et al 1996, Jimenez et al 1997). While naturally occurring OA animal models may most accurately represent the slowly progressing OA that is seen in humans, studying the development of pathogenesis and effectiveness of therapeutics takes a long time (Bendele 2001).

Finally, OA can be induced in animal models through injecting enzymes or chemicals into the articular space. Collagenase is an enzyme that functions to cleave collagen, when collagenase was injected into the knee of mice it was found to cause instability of the joint and subsequent OA-like histological changes such as cartilage lesions and osteophyte formation (van der Kraan et al 1990). Additionally OA can be induced by chemically damaging the joint; an intrarticular injection of monoiodoacetate causes pain-like behavior and destruction of articular cartilage similar to that observed in human OA (Kalbhen 1987). The animal model used to represent knee OA in this thesis is the monoiodoacetate (MIA)model.

5.1.2 The Monoiodoacetate (MIA) model

Monoiodine acetate was first used by Kalbhen and Blum in hens to reduce the anabolic reactions that occur in articular cartilage, an intrarticular injection was found to cause degeneration of joint cartilage, and this finding was extended to rats in 1987 (Kalbhen

and Blum 1977, Kalbhen 1987). Monoiodoacetate (MIA) is a potent inhibitor of the activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is required for glycolysis (Dunham et al 1992, Clements et al 2009). Glycolysis is the first key stage of respiration, and the process used in anaerobic conditions for the cell to create energy. This process allows cells to break down glucose and produce ATP (Engelking 2015). Therefore, MIA prevents chondrocytes in the articular cartilage from producing ATP and leads to their eventual cell death (Guzman et al 2003). Following the loss of chondrocytes, cartilage erosion and a loss of proteoglycan develop, and over time this can result in exposure of the subchondral bone (Guzman et al 2003).

There are many advantages to the MIA model, including the lack of invasiveness and the simplicity of induction, its rapid onset, the pain-like behaviour that develops, and the histological changes that occur in the articular cartilage, which are similar to clinical features observed in human OA. The MIA model is particularly relevant for studying the pain associated with OA as animals display significant pain behaviour; the animals develop sensitivity at both the knee joint and ipsilateral hind paw as they avoid putting weight on the injured limb and show a decreased paw withdrawal threshold (Fernihough et al 2004, Ferland et al 2011, Ogbonna et al 2013). Additionally, micro-CT and histology images have confirmed that MIA injected animals show similar knee pathology to that observed in OA patients, with cartilage fibrillation, loss of proteoglycan, osteophyte formation and subchondral bone sclerosis (Guzman et al 2003, Mohan et al 2011). A further advantage of this model is that the dose of MIA injected into the intrarticular space can be altered to modify the progression, severity of the structural joint damage and the associated pain (Guingamp et al 1997, Schulert and McDougall 2009, Thakur et al 2012). Overall, the MIA model represents the joint pathology and associated pain observed in human OA, and this is the most common method used to model the disease (Little and Zaki 2012).

5.1.2.1 Knee pathology in the MIA model

The injection of MIA into the articular space has been shown to cause an initial inflammatory response, which is followed by structural changes to the joint at later time points (Clements et al 2009). Various histological changes can be observed in the knee joint in both the early inflammatory phase and the later chronic phase, the pathological changes and joint structures affected are dependent upon the dose of MIA used and the chosen time point.

The changes within the joint following MIA injection occur rapidly. The degradation process begins with an inhibition of glycolysis and chondrocyte degeneration, and as such proteoglycan loss can be observed just 1 day following MIA injection (Guzman et al 2003). There is also a significant increase in the levels of monocytes, neutrophils and basophils 1 day after the MIA injection, indicating a large inflammatory infiltrate (Guzman et al 2003, Clements et al 2009). The increased level of white blood cells peaks around day 3 and then begins to decline over time (Clements et al 2009). In addition to the inflammatory changes that occur in the early stages, necrosis of the synovial membrane and oedema of the infrapatellar fat pad can be observed 3 days post-MIA injection (Guzman et al 2003, Clements et al 2009).

At 5 days post MIA injection histological changes in the articular cartilage can be observed, this includes a collapsed or eroded cartilaginous matrix with extensive proteoglycan loss (Dunham et al 1992. Guzman et al 2003, Mohan et al 2011). Although the inflammatory response in the synovium is still present, this has begun to subside (Guzman et al 2003). The subchondral bone begins to show signs of damage at 7 days following a medium to high dose MIA injection, which comprises bone necrosis and an increased number of osteoclasts adjacent to the damaged cartilage (Guzman et al 2003, Clements et al 2009). Overall by day 7-post MIA injection the significant loss of articular cartilage and damage to the subchondral bone begin to reflect the pathology observed in human OA.

At 2-weeks post MIA injection the cartilage damage becomes more severe and extensive, while the proteoglycan content remains greatly reduced (Guingamp et al 1997, Clements et al 2009). Additionally more extensive damage can be observed in the underlying subchondral bone, with bone sclerosis and areas of bone marrow lesions adjacent to the damaged cartilage (Guingamp et al 1997, Guzman et al 2003). Micro-CT imaging has identified that injected joints show a significantly lower bone volume fraction at 2 weeks post injection, even with a low dose of MIA (0.2mg) (Mohan et al 2011). If the model is continued further a complete loss of cartilage from the bone surface is observed, coupled with extensive subchondral bone remodeling that includes bone sclerosis and even bone fragmentation in extreme cases as shown in Figure 5.1 (Guingamp et al 1997, Guzman et al 2003, Mohan et al 2011). The joint pathology induced by an MIA injection worsens over time, with increasing severity in cartilage degradation and subchondral bone damage.

Figure 5.1. Femoro-tibial joint 28 days post 1mg MIA injection. The arrow indicates an extensive area of cartilage loss, while the subchondral bone adjacent to this cartilage damage has begun to collapse and fragment and be replaced by fibrous tissue. (Taken from Guzman et al 2003).

5.1.2.2 Pain-like behaviour in the MIA model

As the focus of this thesis is the pain associated with OA it is important to use an animal model that consistently develops OA-like pain. Following MIA injection animals adjust their gait and shift their weight to the uninjured limb, this is similar to patients who avoid using the injured limb to minimise the associated pain (Bove et al 2003, Zeni and Higginson 2011). The MIA injection has been demonstrated to cause a biphasic shift in weight bearing; with the animals avoiding placing weight on the injured joint on day 3 in line with the peak of inflammation, the joint pain recovers slightly by day 7 with animals applying more weight to the injured limb, and weight bearing then declines again on day 14 when structural damage has developed in the knee (Bove et al 2003, Thakur et al 2012, Kelly et al 2013). The alteration in weight bearing has been shown to remain constant when the duration of MIA model is extended to 6 weeks (Bove et al 2003, Kelly

et al 2013, Ogbonna et al 2015). Additionally, the MIA injection has been shown to increase movement evoked nociception, as MIA animals have higher knee bend scores than controls (Ferreira-Gomes et al 2008). Furthermore, CatWalk analysis has demonstrated that MIA injected animals load less weight on their injured limb when walking and also show significant differences in their dynamic gait between hind limbs indicating the animals walk with a well-defined limp as they are unwilling to place weight on the injured limb (Ferland et al 2011, Ferreira-Gomes et al 2008). These finding indicate that the MIA model accurately mimics the clinical joint pain experienced by patients, as 3D walking analysis has demonstrated that patients with knee OA reduce knee joint contribution when walking (Zeni and Higginson 2011).

In addition to sensitivity within the joint the animals also develop secondary mechanical hyperalgesia and tactile allodynia on the ipsilateral hind paw. The MIA injection induces mechanical hypersensitivity, as demonstrated by a reduction in paw withdrawal threshold to mechanical stimulation, which occurs around 3 days post MIA-injection depending upon the dose, the paw withdrawal threshold then declines further at approximately 14 days and this is sustained for 4 weeks (Ogbonna et al 2013, Pitcher et al 2016). This secondary hypersensitivity may represent the referred pain that develops in many OA patients, indicating the MIA model may prove a useful model for analyzing the involvement of central changes and testing the effectiveness of analgesics (Kean et al 2004, Graven-Nielsen 2006, Gwilym et al 2009).

5.1.2.3 Electrophysiology in the MIA model

One mechanism involved in the development of joint pain is peripheral sensitisation where there is increased activity of primary afferent fibres (See Section 1.1.4). Electrophysiological recordings have indicated that knee joint afferents become sensitised following MIA injection (Schuelert and McDougall 2006, Schuelert and McDougall 2009, Schuelert and McDougall 2012, Kelly et al 2012). Schuelert and McDougall have demonstrated that MIA induces a dose-dependent increase in both spontaneous joint afferent firing frequency and evoked joint afferent activity in response to normal and noxious hyper-rotation of the knee joint (Schuelert and McDougall 2006, Schuelert and McDougall 2009, Schuelert and McDougall 2012). Furthermore, the authors found that the enhanced activity of knee joint afferents could be significantly reduced by NSAIDs, a neuropeptide antagonist, and a $Nav_{1.8}$ channel blocker, indicating this may be a model for analyzing the effectiveness of analgesics

(Schuelert and McDougall 2006, Schuelert and McDougall 2009, Schuelert and McDougall 2012). Furthermore, it was demonstrated that A fibres specifically exhibit an increased spontaneous firing rate while C fibres show an increased evoked firing frequency to mechanical stimulation (Kelly et al 2012). This may indicate that peripheral A fibre joint afferents are responsible for spontaneous pain at rest while C fibres contribute to the increased pain response upon movement, both of which are clinical features reported by OA patients (Kelly et al 2012). These two distinct physiological mechanisms may combine to produce a barrage of peripheral input resulting in spontaneous and evoked joint pain and potentially leading to the development of central sensitization (Woolf 2011).

Electrophysiological recordings taken from spinal neurons in the dorsal horn, which are responsible for transmitting the nociceptive signal from the periphery to the brain, have also demonstrated enhanced firing rates in MIA animals indicative of central sensitization (Rahman et al 2009, Harvey and Dickenson 2009, Sagar et al 2010, Burnham and Dickenson 2013). While most of these studies found that WDR neurons had an increased firing rate in response to mechanical stimulations in the noxious range, Sagar et al found an increased WDR neuronal response to innocuous mechanical stimulation only (Rahman et al 2009, Harvey and Dickenson 2009, Sagar et al 2010, Burnham and Dickenson 2013). Additionally, enhanced WDR neuronal responses have been demonstrated in response to thermal stimulations in the noxious range (Rahman et al 2009, Burnham and Dickenson 2013). This conflicts with an MIA study in mice by Harvey and Dickenson where thermally evoked WDR neuronal responses were not significantly different from shams and no thermal hypersensitivity was detected behaviourally (Harvey and Dickenson 2013). Furthermore, two thesis's produced by the lab reported no alteration in either the mechanically or thermally evoked responses of WDR neurons located in the deep dorsal horn between MIA and Sham groups (Thakur 2012, Patel 2012). Finally, it has been reported that WDR neurons receiving input from both the knee and hind paw in MIA animals have an enhanced spontaneous firing rate compared to Shams (McGaraughty et al 2010, Chu et al 2011). The lack of thermal hypersensitivity in this model is in agreement with clinical findings, a study using quantitative sensory testing (QST) in patients with chronic knee OA showed that only 7% of patients displayed thermal hyperalgesia while 37% of patients displayed mechanical hyperalgesia, the authors subsequently used mechanical hyperalegsia as a marker to define central sensitization in patients yet did not use thermal hyperalgesia (Hochman et al 2013). While the contrasting electrophysiological observations in MIA

animals are difficult to explain and may come down to inter-experimenter differences, they do represent the variability in clinical pain symptoms found in OA patients (Gwilym et al 2009).

A further clinical feature of central sensitisation in OA patients is the development of referred pain distant to the site of joint damage (Bajaj et al 2001, Gwilym et al 2009). WDR spinal neurons in animal models of both chronic neuropathy and inflammation have demonstrated significantly expanded receptive fields from the hind paw (Neugebauer et al 1993, Chu et al 2004). In MIA animals, WDR neurons receiving input from the knee showed an expansion in the peripheral receptive field of the knee following an intra-articular injection of NGF, a neurotrophin implicated in the inflammatory component and development of chronic pain in OA (Sagar et al 2015). The expansion of the receptive field means second order neurons in the spinal cord now receive input from a wider array of afferent fibres and will subsequently produce an increased response to peripheral stimulation, this indicates the involvement of central plasticity contributing to hypersensitivity (Graven-Neilsen 2006).

Taken together these electrophysiological findings indicate that the initial peripheral damage and enhanced activity of afferent fibres produces a barrage of nociceptive signaling to second order spinal neurons, which may drive the increased activity of central neurons and the development of central sensitisation. Therefore, the spontaneous and movement-evoked chronic pain profile observed in MIA animals may be due to a combination of both peripheral and central hypersensitivity.

5.1.2.4 Descending controls in the MIA model

Many patients are left with persistent pain even following total joint replacement surgery, indicating that pain remains even when the peripheral nociceptive input is removed (Wylde et al 2011). An alteration in descending controls and supraspinal systems, together with central sensitisation and the development of referred pain may be responsible for this persistent pain. Indeed, a clinical study using fMRI identified an abnormal descending system from the PAG to the spinal cord in patients with hip OA and referred pain (Gwilym et al 2009). An adaptive descending system has also been identified in the MIA model (Rahman et al 2009, Li et al 2011, Burnham and Dickenson 2013). Firstly, Rahman and colleagues found that a 5-HT_3 receptor antagonist inhibited evoked responses to innocuous stimuli yet did not produce this effect in Sham controls,

indicating an alteration in the faciliatorty serotonergic system acting at 5-HT_3 receptors in the spinal cord was modulating low threshold neuronal responses (Rahman et al 2009). Meanwhile, Li and colleagues found a 5-$HT_{2A/C}$ receptor antagonist blocked the analgesic effect of electroacupuncture in MIA animals, and suggested that electroacupunture mediated its inhibitory effect by activating serotonergic neurons in the NRM, which projected to the spinal cord and activated 5-HT_2 receptors (Li et al 2011). These contrasting serotonergic findings further suggest that the adaptive serotonergic system can switch from an antinocicieptove to pronociceptive role depending upon which receptors are activated in the spinal cord. Furthermore, a reduced inhibitory noradrenergic descending system has been demonstrated in MIA animals (Burnham and Dickenson 2013). As a subset of OA patients have displayed reduced descending inhibition and an alteration in descending systems from the brainstem, similar alterations in the MIA model suggest this may prove a useful tool for assessing the effectiveness of therapeutics that act to modulate the endogenous descending system (Gwilym et al 2009, Ardendt-Nielson et al 2010).

5.1.3 Chapter aims

This chapter will use both behavioural and histological techniques to validate the 2mg MIA model in the rat. Furthermore, the alteration in descending facilitatory and inhibitory systems shall be investigated through assessing DNIC in the MIA model. As variations in the activity of inhibitory and faciliatory pathways have been demonstrated over the course of the MIA model, DNIC shall be investigated in both the early inflammatory phase and late chronic phase of the model (Burnham and Dickenson 2013).

5.2 Methods

5.2.1 Electrophysiology

Electrophysiological recordings were taken as previously described (See Section 2.1); in early phase animals this was 2-6 days post MIA injection, and for late phase animals this was 14-20 days post MIA injection. Extracellular recordings were taken from WDR neurons both ipsilateral and contralateral to MIA injection.

5.2.1.1 Characterisation of DNIC expression in WDR neurons in early and late Phase MIA animals

In MIA injected and saline injected Sham animals, WDR neurons were found in both the ipsilateral and contralateral deep dorsal horn relative to injection, and the DNIC response was characterized. The receptive field was mechanically stimulated with von Frey hairs of increasing intensity: 8g, 26g and 60g, this was repeated 3 times to obtain an average pre-conditioned response. DNIC was then induced with a concurrent noxious ear pinch as the receptive field was mechanically stimulated with the von Frey hairs of increasing intensity. For this study DNIC was also induced with a concurrent noxious knee pinch. For the noxious knee pinch a metal clamp was used that was wrapped around the knee and the two sides were pushed together, the clamp was set to always pinch at 0.75cm. Both the MIA injected knee and the contralateral uninjured knee received a concurrent noxious pinch. Therefore, one DNIC trial consists of 3 pre-conditioned mechanically evoked responses, 1 mechanically evoked response with concurrent noxious ear pinch, 1 mechanically evoked response with concurrent ipsilateral noxious knee pinch and 1 mechanically evoked response with a concurrent contralateral noxious knee pinch. Each characterization consisted of 2 DNIC trials and the mean of these two trials was taken for the pre-conditioned and DNIC responses.

5.2.2 Behavioural assessment

Both paw withdrawal thresholds and weight bearing between the animals hind paws were assessed as described in Section 2.4.

5.2.3 Knee Histology

Knee histology was carried out in early phase MIA animals and saline injected sham controls, where the knee was taken on day 4 post-injection. Knee histology was carried

out in late phase MIA animals and saline injected sham controls, where the knee was taken on day 14 post-injection. The tissue was processed as discussed in Section 2.5.

5.2.4 Quantitative Polymerase Chain Reaction

The ipsilateral lumbar dorsal horn and ipsilateral L3-L5 DRGs were taken from MIA and sham animals. For early phase MIA and sham animals the tissue was taken 4 days post-injection. For late phase MIA and sham animals the tissue was taken 14 days post-injection. The tissue was processed as discussed in Section 2.7.

5.2.3 Statistical analysis

For a description of the statistical tests used for analysis please refer to section 2.8.

5.3 Results

5.3.1 Pain-like behaviour in the MIA model

Pain-like behaviour was assessed by testing for puncate mechanical hypersensitivity and incapacitance in MIA-injected rats and compared to Sham controls. Both groups of MIA injected animals, the early inflammatory phase and late chronic phase, developed mechanical hypersensitivity on the ipsilateral hind paw and avoided putting weight on the injured limb.

Early phase MIA-injected animals demonstrated a significant reduction of weight borne on the MIA injected limb, such that on day 2 they only placed 28.17% ± 2.7% and on day 4 they only placed 35.35% ± 2.14% of their distributed weight between the two hind limbs on the injured paw (Figure 5.2A). On day 2 the early phase MIA-injected animals placed significantly less weight on the injured limb compared to saline-injected sham controls at the same time point. Although the weight placed on the hind paw by early phase MIA-injected animals was reduced at day 4 compared to saline-injected shams this did not reach significance (Figure 5.2C). Late phase MIA-injected animals also demonstrated a significant reduction in the weight placed on the MIA injected limb. MIA-injected animals in the late phase group placed 24.54% ± 2.72%, 32.34% ± 2.28%, 33.5% ± 1.85%, 32.34% ± 2.31% of their weight on the injured limb on day 2, 4, 7 and 14 respectively (Figure 5.2D). At all time points, other than on day 4, the MIA-injected animals in the late phase group placed a significantly reduced amount of weight on the injured limb compared to saline-injected sham controls at the same time points (Figure 5.2F).

To begin with, testing how often the rat withdrew its paw to a specific von Frey hair out of 5 trials was the method used for assessing punctate mechanical hypersensitivity, and the paw withdrawal threshold was determined as the point at which the animal withdrew 5 times. This test revealed that early phase MIA animals had a significantly reduced paw withdrawal threshold on the ipsilateral paw at day 2 compared to saline-injected sham controls (Figure 5.3A). While the early phase MIA animals withdrew more frequently to 2g, 8g, and 15g von Frey hairs compared to saline-injected sham controls, this was only significant for 2g on day 2 and 15g on day 2 and 4 (Figure 5.3C and 5.3G). The up-down method was also used to assess any changes in the paw withdrawal thresholds, this test revealed that early phase MIA animals had a significantly reduced

paw withdrawal threshold on the ipsilateral paw at day 2 compared to saline-injected sham controls. Both tests indicate that early phase MIA animals develop mechanical hypersensitivity at day 2 but recover slightly around day 4. No mechanical hypersensitivity was observed in the contralateral hind paw in early phase MIA or Sham animals with either behavioural method used (Figure 5.3).

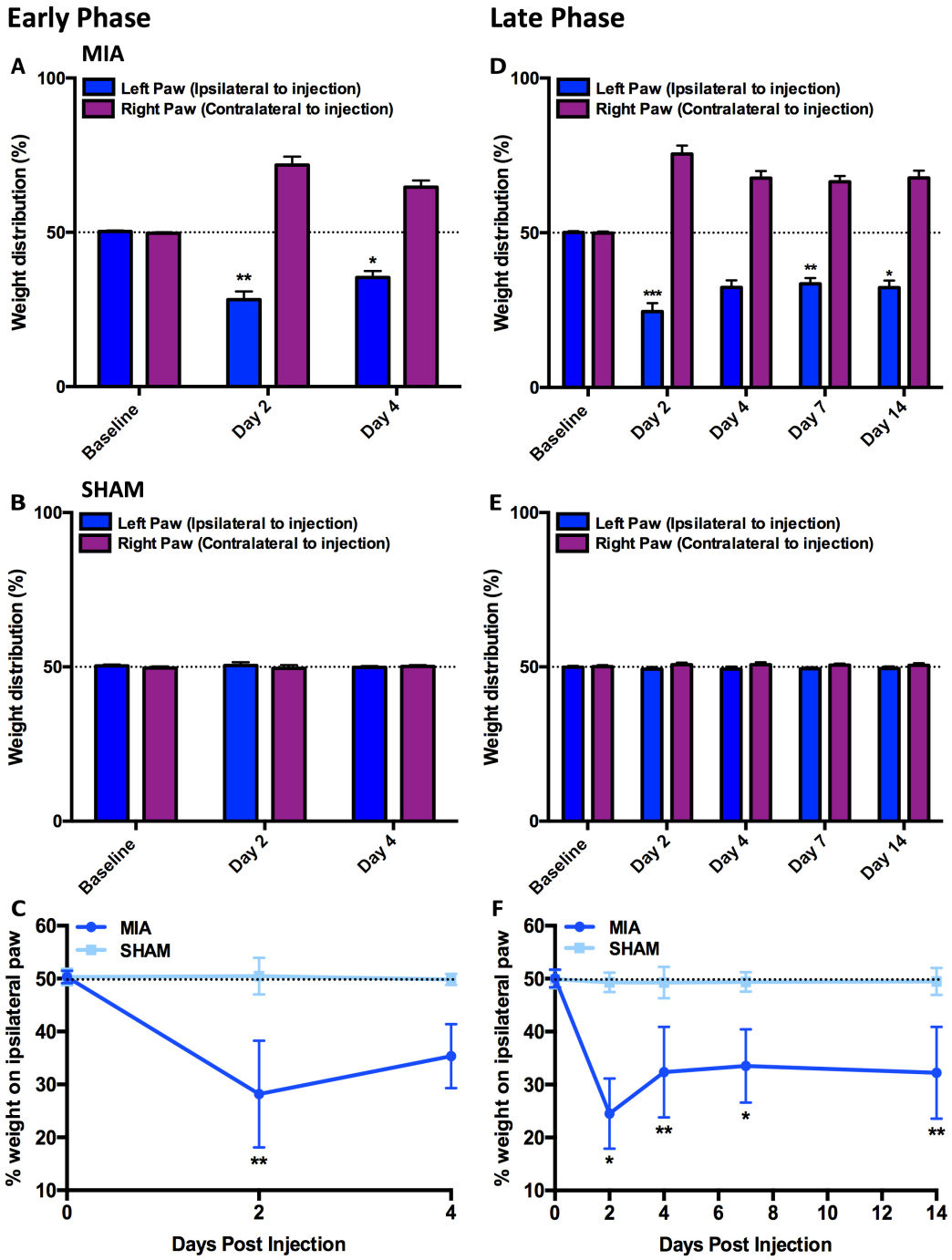


Figure 5.2. Weight bearing in early phase and late phase MIA-injected animals compared to saline-injected sham controls. A) Early phase MIA-injected animals placed significantly less weight on the injured joint (n=15). B) Saline-injected sham controls consistently placed similar

amounts of weight on both hind paws (n=12). C) Early phase MIA injected animals placed less weight on the ipsilateral paw compared to saline-injected sham controls and this was significant at day 2. D) MIA-injected animals in the late phase groups placed significantly less weight on the injured joint at every time point other than day 4 (n=14). E) Saline-injected sham controls consistently placed similar amounts of weight on both hind paws at the same times points tested for late phase animals (n=14). E) MIA-injected animals in the late phase group placed significantly less weight on the ipsilateral hind paw compared to saline-injected sham controls at all time points tested. A Friedmans two-way ANOVA by ranks test was used to assess significant differences in weights placed between hind paws. A Kruskal-Wallis independent samples one-way Anova was used to assess significant differences in weight placed on the ipsilatral paw between MIA-injected animals and saline-injected sham controls. *P<0.05, **P<0.01.

Behavioural tests were also used to assess the development of mechanical hypersensitivity in late phase MIA animals and saline-injected sham controls. The paw withdrawal threshold determined by the animal withdrawing 5 times demonstrated that MIA injected animals have a significantly reduced paw withdrawal threshold at days 2, 4 and 14 (Figure 5.4A). The MIA injected animals in the late phase group withdrew more frequently to the 2g vonFrey hair compared to saline-injected controls and this was significant on day 2 and 14 (Figure 5.4C), significantly more frequently to the 8g vonFrey hair compared to saline-injected controls on day 2, 4 and 14 (Figure 5.4E), and significantly more frequently to the 15g vonFrey hair compared to saline-injected controls on day 4, 7 and 14 (Figure 5.4G). The up-down method also indicated that MIA injected animals develop mechanical hypersensitivity as they had a significantly reduced paw withdrawal threshold compared to saline-injected sham controls at all time points tested (Figure 5.4I). Taken together these results indicated that the MIA animals in the late phase group developed bi-phasic mechanical hypersensitivity on the paw ipsilateral to injection. The data demonstrate that mechanical hypersensitivity becomes apparent by day 2, appears to recover slightly around day 4 and 7 as paw withdrawal threshold increases and paw withdrawal frequency decreases although for some tests and von Frey hairs tested there is still a statistically significant difference between MIA injected animals and saline-injected controls at these time points, and then all tests indicate mechanical hypersensitivity returns again at day 14. Similarly to early phase animals there is no mechanical hypersensitivity observed on the contralateral paw with any of the behavioural methods tested (Figure 5.4).

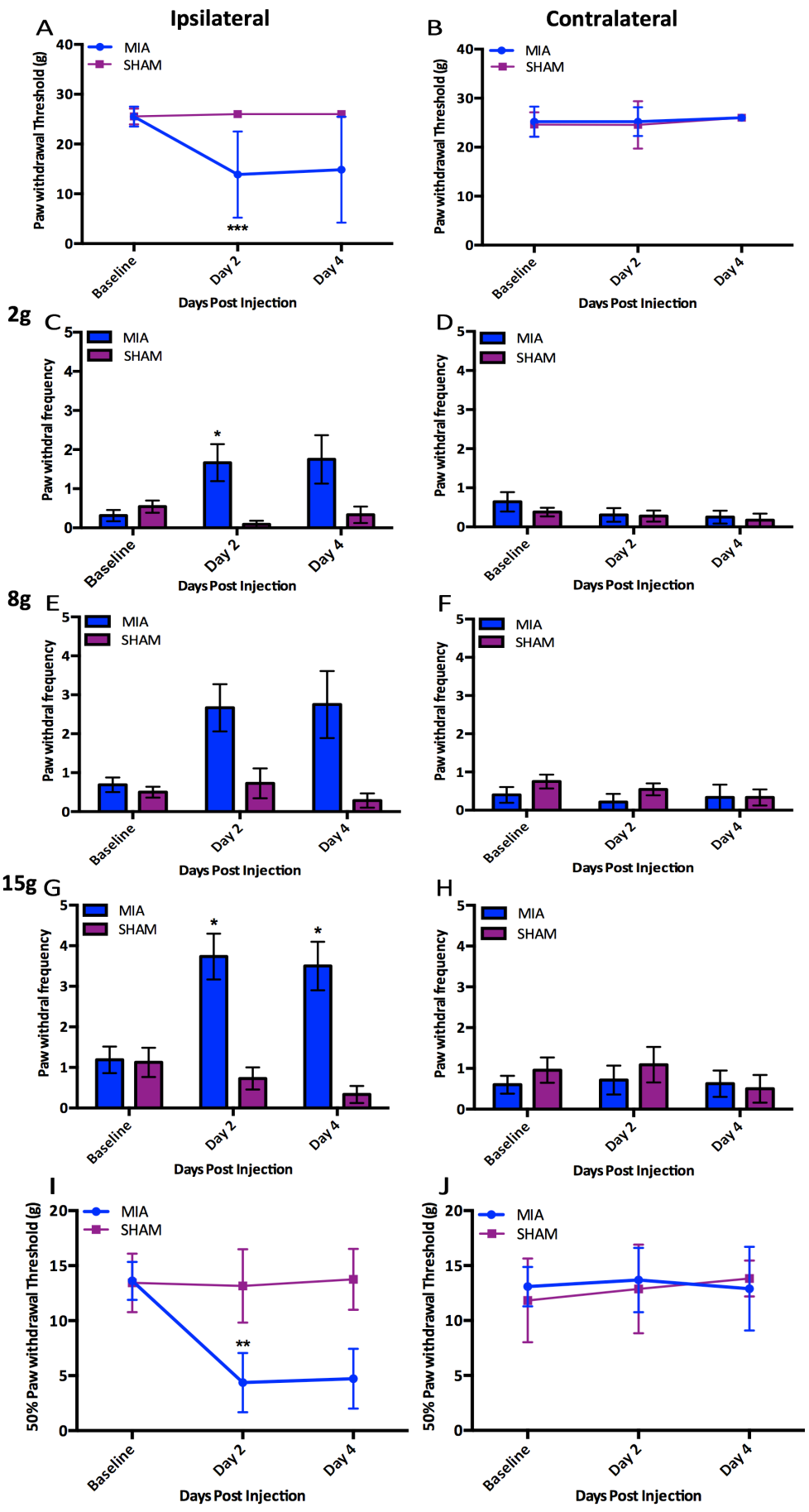

Ipsilateral
Contralateral
A
B
C
D
E
F
G
H
I
J
2g
8g
15g
MIA
SHAM
Paw withdrawal Threshold (g)
50% Paw withdrawal Threshold (g)
Paw withdral frequency
Baseline
Day 2
Day 4
Days Post Injection
***
*
**

Figure 5.3. Mechanical hypersensitivity in early phase MIA animals. A) The paw withdrawal threshold was determined by the animal withdrawal withdrawing 5 times to that von Frey hair. This method indicates that early phase MIA animals have a significantly reduced withdrawal threshold on the ipsilateral paw to injection on day 2 (EP n=16, EPS n=12). B) The MIA-injected animals and saline-injected sham controls displayed no significant changes in their withdrawal threshold on the contralateral hind paw. C) The MIA-injected animals withdraw their paw more frequently to a 2g von Frey hair, this is significant at day 2. D) There is no significant difference in the number of withdrawals on the contralateral hind paw between MIA-injected and saline injected animals. E) The MIA injected animals withdraw more frequently to an 8g von Frey hair yet this is not significant. F) There is no significant difference in the number of withdrawals on the contralateral hind paw between MIA injected animals and saline injected sham controls. G) There is a significant increase in the number of withdrawals to a 15g von Frey hair in the MIA injected animals on day 2 and day 4 compared to saline injected sham controls. H) There is no significant difference in the number of withdrawals with the hind paw contralateral to injection between MIA injected animals and saline injected sham controls. I) The up-down method for assessing mechanical hypersensitivity demonstrates that early phase MIA injected animals have a significantly reduced paw withdrawal threshold on day 2 compared to saline injected sham controls (EP n=10, EPS n=10). J) The up-down method also shows that no mechanical hypersensitivity develops in the contralateral paw to injection as there is no significant difference in the paw withdrawal threshold between MIA injected animals and saline injected sham controls. Kruskall-wallis independent samples one-way ANOVA. *P<0.05, **P<0.01, ***P<0.001.

5.3.2 Histology in the MIA model

Knee histology was performed to assess any damage to the cartilage in the knee caused by the MIA injection. The maximum knee histology score represents the point of greatest damage while the maximum per slide average score represents the cartilage damage throughout the knee. Both of these scoring methods demonstrate that that cartilage in the MIA injected animals in the late phase group was significantly damaged compared to saline-injected controls (Figure 5.5A, 5.5B and 5.5D). Remarkably MIA injected animals in the early phase group displayed very little cartilage damage, in fact there was no significant difference in knee scores between early phase MIA animals and early phase sham animals with either scoring method (Figure 5.5A, 5.5B and 5.5C). There was little to no damage in the cartilage of early phase and late phase saline-injected sham controls (Figure 5.5E and Figure 5.5F). There was no significant difference in the level of cartilage damage between the four knee condyles in either early phase or late phase MIA injected animals, suggesting the MIA is evenly dispersed through the intrarticular space (Figure 5.5G and 5.5H). These results indicate that the early phase MIA injected animals may not accurately represent the knee pathology of osteoarthritis, while the late phase MIA injected animals display a significant destruction of cartilage similar to that observed in patients.

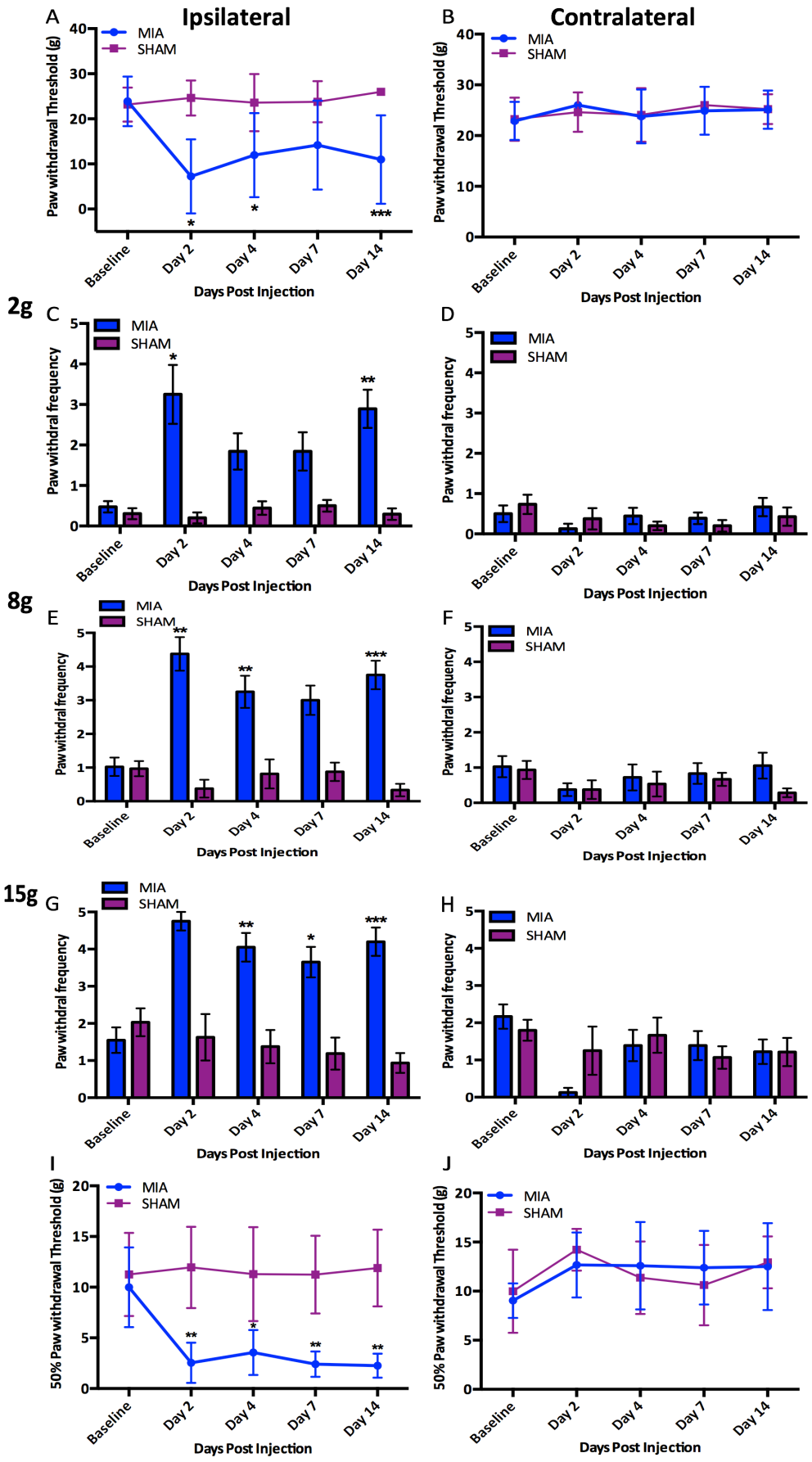

A
Ipsilateral
B
Contralateral
MIA
SHAM
Paw withdrawal Threshold (g)
Baseline
Day 2
Day 4
Day 7
Day 14
Days Post Injection
2g
C
D
Paw withdral frequency
8g
E
F
15g
G
H
I
J
50% Paw withdrawal Threshold (g)

Figure 5.4. Mechanical hypersensitivity in late phase MIA animals. A) The paw withdrawal threshold was determined by the animals withdrawing 5 times to a specific vonFrey hair. This behaviour method demonstrates that MIA injected animals develop a significantly reduced paw withdrawal threshold at the ipsilateral hind paw to injection on day 2 and day 14 post-injection compared to saline injected sham controls (LP n=20, LPS n=15). B) There was no change in paw withdrawal threshold on the contralateral hind paw between MIA injected animals and saline injected sham controls. C) The MIA injected animals withdrew their ipsilateral hind paw to the 2g von Frey hair significantly more times at day 2 and day 14. D) There is no change in the number of withdrawals to 2g on the contralateral hind paw. E) The MIA injected animals withdraw the ipsilateral hind paw to an 8g von Frey hair more frequently on day 2, 4 and 14 compared to saline injected sham controls. F) There was no change in the number of withdrawals to an 8g von Frey hair on the contralateral hind paw to injection in either MIA injected animals or saline injected sham controls. G) The MIA injected animals withdrew their ipsilateral hind paw significantly more frequently to a 15g von Frey hair at all time points compared to saline injected controls. H) There is no change in the number of withdrawals to a 15g von Frey hair on the contralateral hind paw to injection in either MIA injected animals or saline injected sham controls. I) The up-down method to assess mechanical hypersensitivity demonstrates that MIA injected animals develop a significantly reduced paw withdrawal threshold at all time points tested following injection compared to saline injected sham controls (LP n=10, LPS n=13). J) The up-down method demonstrated there was no significant difference in the paw withdrawal threshold of the contralateral hind paw to injection following MIA or saline injection. Kruskal-wallis independent samples one-way ANOVA. *P<0.05, **P<0.01, ***P<0.001.

5.3.3 Correlation between cartilage damage and pain-like behaviour in late phase MIA animals

The MIA model has faced criticism that while it represents the symptoms associated with the development of OA, especially pain, it may not accurately model the structural changes that occur within the human joint (Little and Zaki 2012). It has also been suggested that aspects of the pain phenotype may be attributed to the neurotoxicity of the MIA destroying other tissues surrounding the joint (Thakur et al 2012, Malfait et al 2013). The results from early phase MIA animals agree with this concept as rats development significant pain-like behaviour despite the absence of any joint pathology (Figure 5.5). However, significant cartilage destruction was observed in late phase MIA animals, and a correlation between the level of cartilage damage and the pain-like behaviour on day 14 was assessed. A negative correlation exists between knee scores and weight bearing such that the less weight placed on the ipsilateral paw the higher knee histology score was observed (Figure 5.6A and B). The correlation was only significant for weight bearing v maximum average knee histology score suggesting the more cartilage damage that occurred throughout the entire joint the more animals avoided placing weight on that limb (Figure 5.6B). There was also a negative correlation between knee histology scores and paw withdrawal thresholds such that animals with lower paw withdrawal thresholds had higher knee scores, suggesting increased mechanical hypersensitivity developed on the ipsilateral hind paw on day 14 when there was extensive cartilage damage (Figure 5.6C and D).

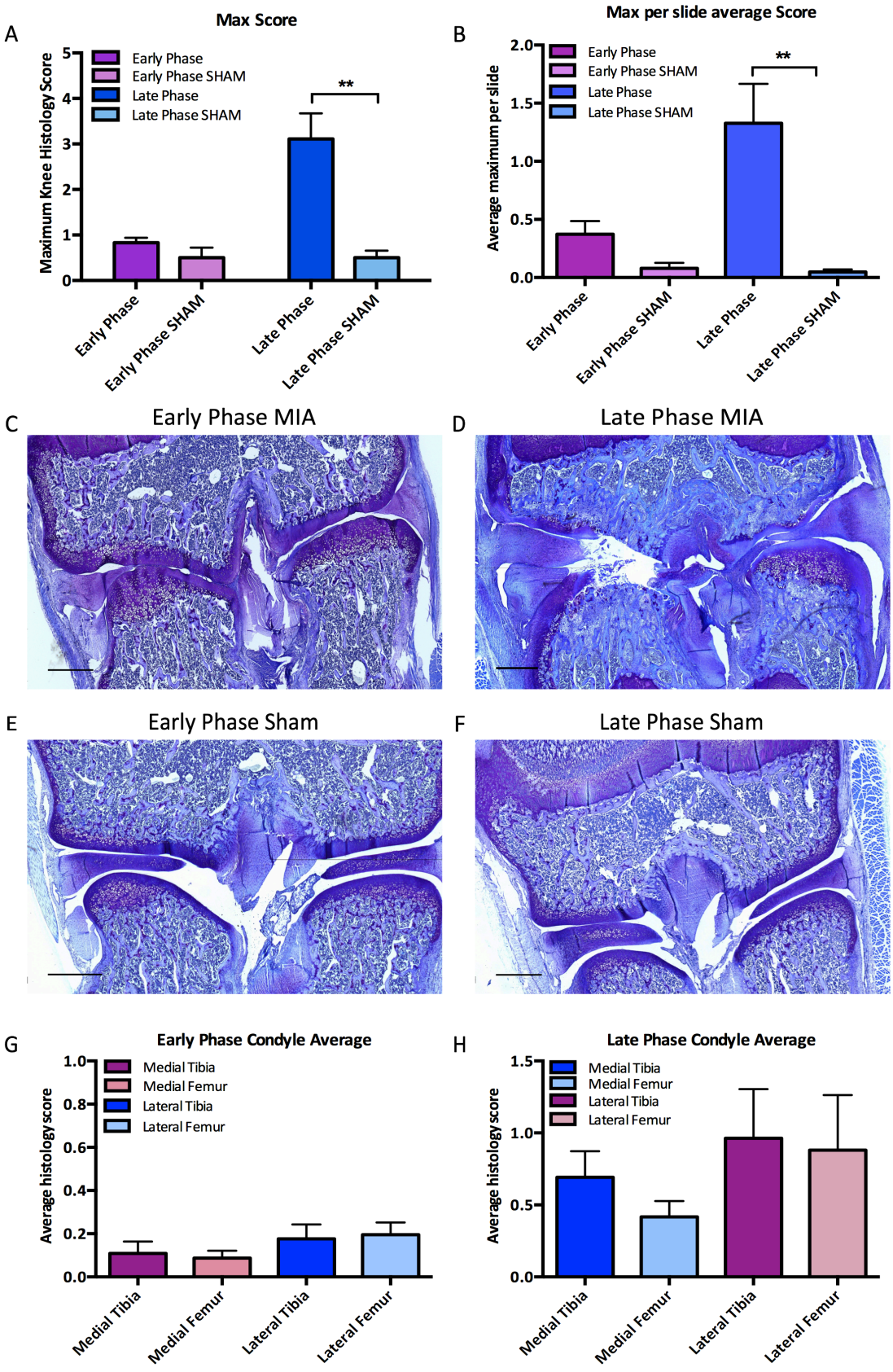


Figure 5.5. Knee histology in MIA animals. A) For maximum knee histology score the maximum score from each knee was selected. MIA injected animals in the early phase group do not demonstrate damaged cartilage compared to saline injected sham controls, while MIA

injected animals in the late phase group had significantly degraded cartilage compared to saline-injected sham controls. B) For the max per slide average score the highest score on every fourth slide was taken and averaged to obtain the level of cartilage damage throughout the knee. This scoring system demonstrated no significant cartilage damage between early phase MIA injected animals and saline injected sham controls. Meanwhile, the MIA injected animals in the late phase group had significantly damaged cartilage throughout the knee compared to the saline injected controls. C) An example image taken from knee histology from an early phase MIA injected animal, this demonstrates that little cartilage damage can be observed. D) Knee histology from a late phase MIA injected animal, this demonstrates extensive cartilage damage on two condyles such that most of the cartilage has been degraded and subchondral bone is almost exposed. E) Knee histology from a saline injected sham control taken at the same time point as early phase animals, this demonstrates that no cartilage damage can be observed. F) Knee histology from a saline injected sham control taken at the same time point as late phase animals, this demonstrates a lack of cartilage damage in these animals. G) The average histology scores on each condyle in early phase MIA injected animals show no significant difference in the damage of any condyles. H) The average histology scores per condyle from late phase MIA injected animals show there is no significant damage between the level of cartilage damage on each condyle. A Kruskall-wallis independent samples one-way ANOVA was used to test significance differences in knee scores between groups. A Friedmans two-way ANOVA by ranks was used to test for significant differences in knee scores between condyles. **P<0.01. (EP n=6, EPS n=6, LP n=9, LPS n=5).

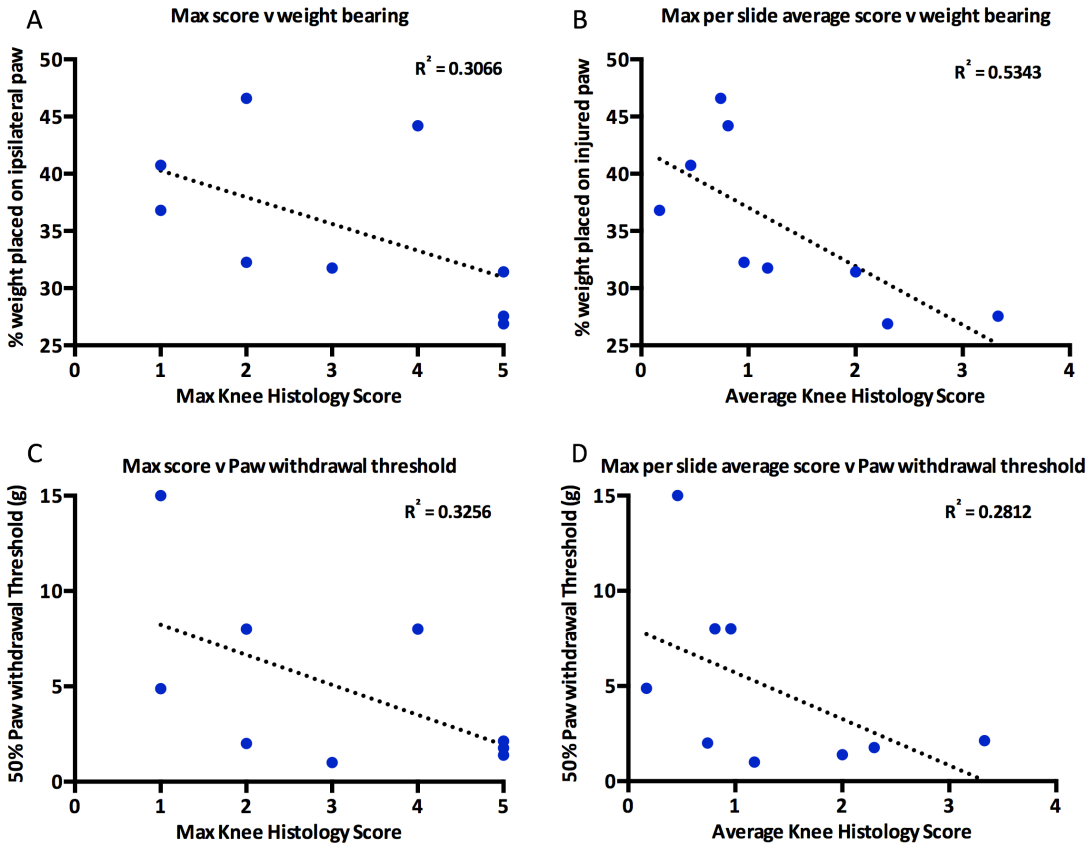


Figure 5.6 Correlation between cartilage damage and pain-like behaviour in late phase MIA animals. A) Late phase MIA animals with higher maximum knee histology schools put less weight on the ipsilateral hind limb, but this correlation does not reach significance (n=9) B) There is a significant negative correlation between the average knee histology score and weight placed on the ipsilateral hind paw in late phase MIA injected animals (n=9). C) Late phase MIA animals with higher maximum knee histology score have reduced paw withdrawal thresholds but this correlation is not significant (n=9). D) Late phase MIA injected animals with higher

average knee scores have lower paw withdrawal thresholds but this correlation is not significant (n=9). Linear regression analysis was performed to assess correlation, R^2 value represent strength of relationship and P value calculated from an F-test of overall significance. A: p=0.1219, B: p=0.0253, C: p=0.1086, D: p=0.1420.

5.3.4 Electrophysiology in the MIA model

Electrophysiological recordings were than taken from WDR neurons located in the deep dorsal horn receiving input from the hind paw ipsilateral to the MIA injection. The hind paw was stimulated mechanically with both innocuous and noxious von Frey hairs of increasing bending force. The mechanically evoked firing rates were not significantly enhanced in MIA injected animals in either the early or late phase groups compared to saline injected sham controls (Figure 5.7).

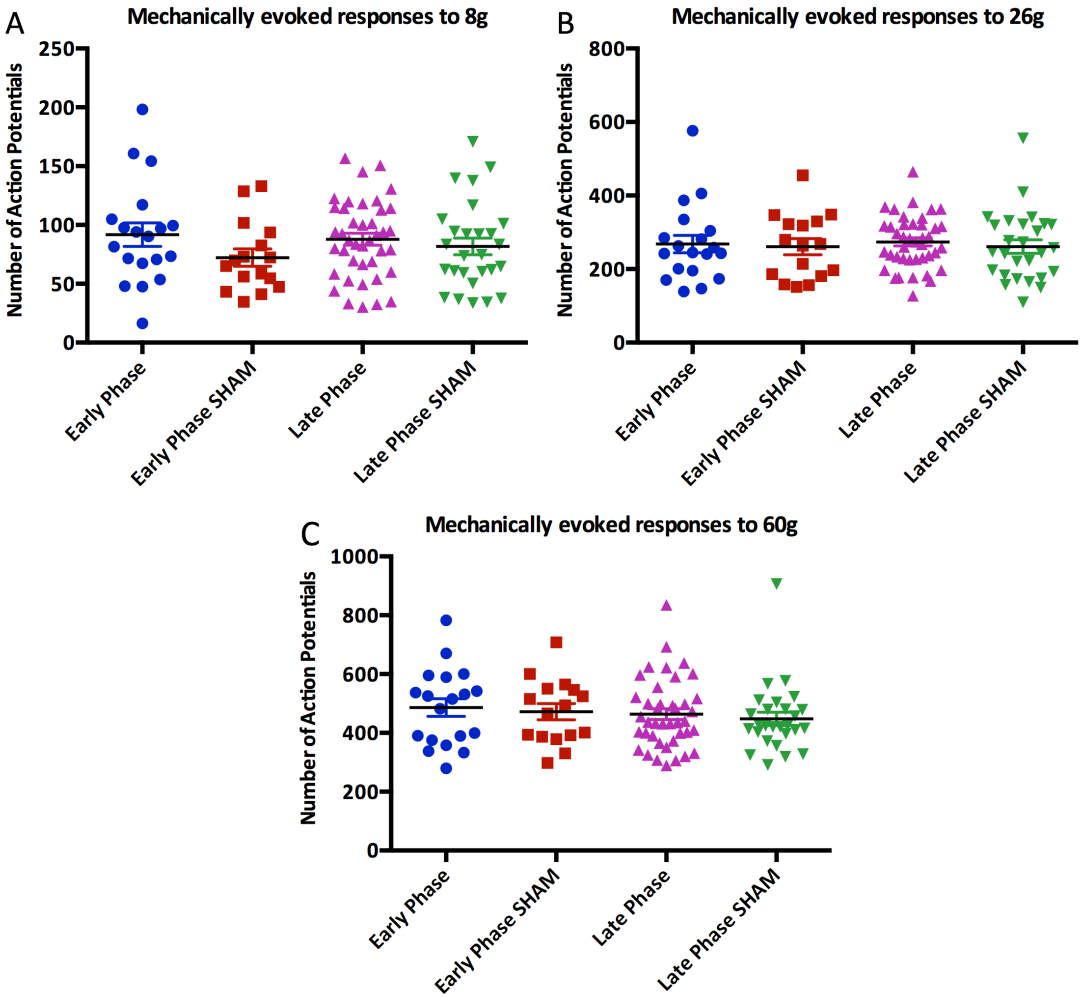


Figure 5.7. The mechanically evoked neuronal responses in early and late phase MIA animals compared to saline injected sham controls. There is no significant difference between the mechanically evoked WDR neuronal responses between MIA and Sham animals in response to 8g (A), 26g (B), and 60g (C) von Frey hair stimulation of the hind paw. (Early phase MIA n=19, Early phase sham n=16, Late phase MIA n=41, Late phase sham n=27). One-way ANOVA with Bonferroni post-hoc test.

5.3.5 Expression of Diffuse Noxious Inhibitory Controls in the MIA model

Throughout this chapter DNIC was induced by a concurrent noxious ear pinch ipsilateral to the WDR neuron being recorded and by a concurrent noxious knee pinch. The concurrent noxious ear pinch produced a significant inhibition of neuronal firing in early phase MIA injected animals and saline injected sham controls. The level of neuronal inhibition produced with noxious ear pinch in early phase MIA animals was 44%, 31%, and 33% for 8g, 26g, and 60g respectively (Figure 5.8A), while the level of neuronal inhibition produced by noxious ear pinch in early phase sham animals was 48%, 31%, and 30% for 8g, 26g, and 60g respectively (Figure 5.8D). A concurrent noxious pinch placed on the injected knee produced a significant reduction of neuronal firing in early phase MIA and saline injected controls. The level of neuronal inhibition produced by the concurrent noxious knee pinch in early phase MIA animals was 54%, 34%, and 41%, for 8g, 26g, and 60g respectively (Figure 5.8A), while the level of neuronal inhibition produced by a concurrent noxious knee pinch in early phase sham animals was 51%, 39%, and 38% for 8g, 26g, and 60g respectively (Figure 5.8D). There was a significant difference between pre-conditioned neuronal firing rates and neuronal firing rates with concurrent noxious ear and knee pinch in both early phase MIA and sham animals at all von Frey weights tested. Furthermore, the level of inhibition produced by the concurrent noxious ear pinch and concurrent noxious knee pinch were comparable between early phase MIA and sham animals.

Electrophysiological recordings were also taken from WDR neurons in the contralateral dorsal horn to injection and the expression of DNIC was investigated with a concurrent noxious ear pinch and a concurrent noxious knee pinch placed on both the injected and uninjured knee. While all of these methods produced an inhibition on neuronal firing in both early phase MIA and sham animals, this was only significant with a concurrent noxious ear pinch with the mechanical stimulation of 26g and 60g in early phase MIA animals (Figure 5.8B), and with a concurrent noxious knee pinch on the uninjured knee with the mechanical stimulation of 60g in sham animals (Figure 5.8E). With a concurrent noxious ear pinch the level of inhibition on neuronal firing in early phase MIA animals was 61%, 33%, and 33% for 8g, 26g, and 60g respectively, while in early phase sham animals the concurrent noxious ear pinch produced a 54%, 34%, and 40% reduction in neuronal firing for 8g, 26g and 60g respectively (Figure 5.8B and 5.8E). In early phase MIA injected animals the concurrent noxious knee pinch placed on the uninjured knee produced a 54%, 40%, and 35% reduction on neuronal firing for 8g, 26g

and 60g respectively, while this produced a 42%, 38%, and 41% reduction of neuronal firing for 8g, 26g and 60g respectively in sham animals (Figure 5.8B and 5.8E). In early phase MIA animals the concurrent noxious knee pinch placed on the injected knee produced a reduction in neuronal firing rate of 55%, 39%, and 30% for 8g, 26g and 60g respectively, with in early phase sham animals the concurrent noxious injected knee pinch produced a 52%, 53%, and 38% reduction in neuronal firing for 8g, 26g, and 60g respectively. Overall, the levels of inhibition on mechanically evoked neuronal firing of WDR neurons in the contralateral dorsal horn were comparable between early phase MIA and sham animals for all methods used to induce DNIC. These findings indicate that the endogenous inhibitory system functions as normal in the early phase MIA animals.

Electrophysiological recordings were also taken from WDR ipsilateral to injection in late phase MIA and sham animals (at least 14 days post-injection). DNIC was abolished in late phase MIA animals; there was no reduction in mechanically evoked neuronal firing with a concurrent noxious ear pinch (Figure 5.9A). Yet remarkably, when the concurrent noxious pinch was placed on the MIA injected knee there was a significant reduction in mechanically evoked neuronal firing (Figure 5.9A). The level of inhibition on neuronal firing produced by the noxious knee pinch was 10%, 18% and 17% for 8g, 26g and 60g mechanically evoked responses respectively. In late phase saline injected sham controls both concurrent noxious ear and injected knee pinch produced a significant reduction in mechanically evoked neuronal firing (Figure 5.9D). In late phase sham animals the level of inhibition on neuronal firing with concurrent noxious ear pinch was 45%, 38%, and 34% for 8g, 26g, and 60g respectively, while concurrent noxious knee pinch produced a 51%, 39%, and 37% reduction on neuronal firing for 8g, 26g, and 60g respectively (Figure 5.9D). Therefore, although the concurrent noxious MIA knee pinch produces an inhibitory effect on neuronal firing in late phase MIA animals it is at much lower levels compared to saline injected sham controls. Taken together these results indicate an alteration in the endogenous inhibitory system that is utilized by DNIC in late phase MIA animals.

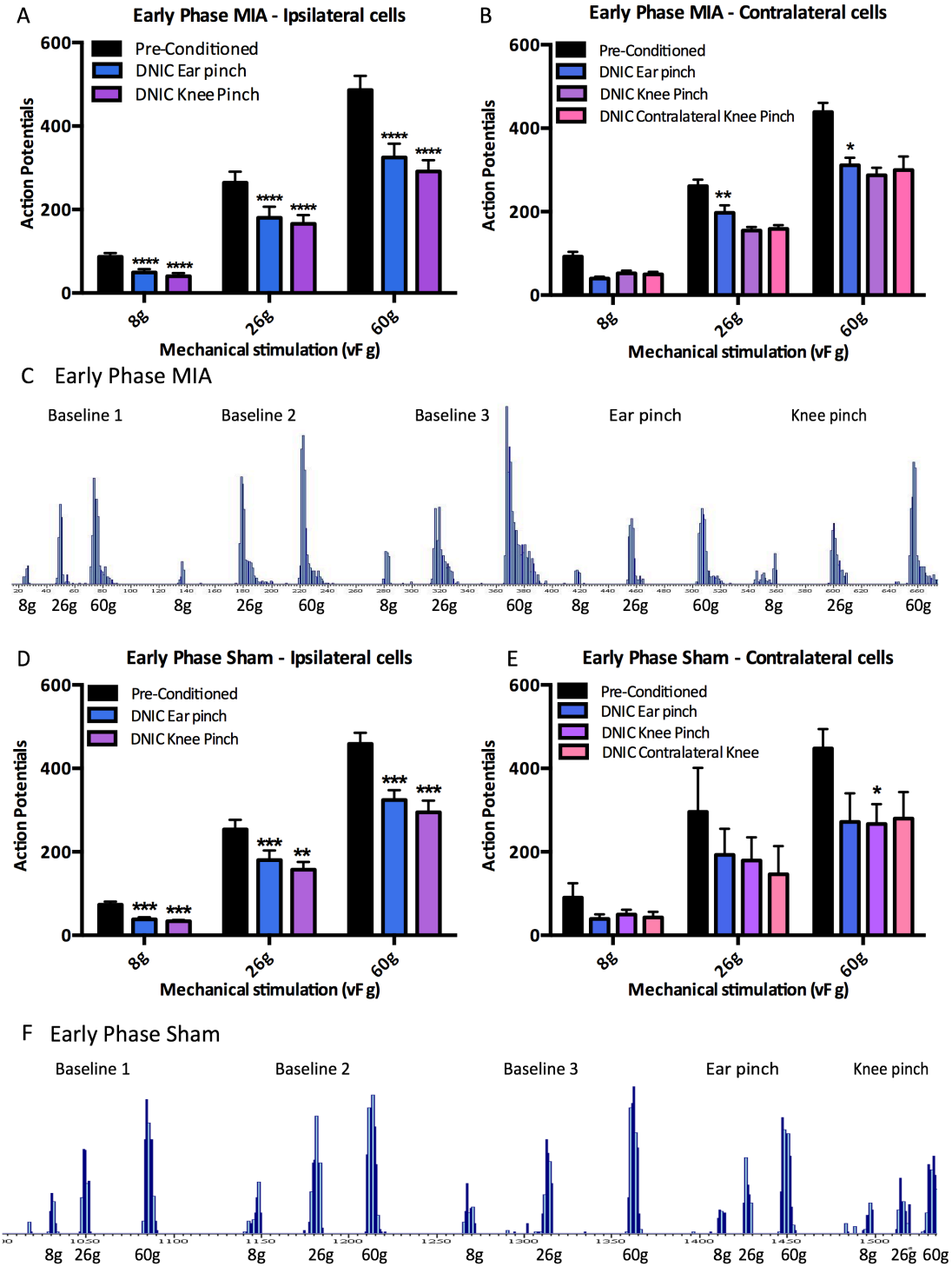


Figure 5.8. The expression of DNIC in early phase MIA injected animals and saline injected sham controls. A) Electrophysiological recordings were taken from WDR neurons ipsilateral to injection in early phase MIA animals. Both concurrent noxious ear and knee pinch produced a significant reduction in mechanically evoked neuronal firing (n=16). B) Electrophysiological recordings were also taken from WDR neurons contralateral to injection. In early phase MIA animals, concurrent noxious ear and injured and uninjured knee pinch reduced mechanically evoked neuronal firing but this was only significant with noxious ear pinch at 26g and 60g (n=5) C) A raw trace from one example ipsilateral WDR neuron in an early phase MIA animal, demonstrating three pre-conditioned baselines and a reduction in mechanically evoked neuronal firing with concurrent noxious ear pinch and injected knee pinch. D) In early phase sham animals a concurrent noxious ear and knee pinch produced a significant reduction in mechanically

evoked neuronal firing (n=16). E) Electrophysiological recordings were taken from contralateral WDR neurons in saline injected sham controls. A concurrent noxious ear, uninjured knee, and injected knee pinch reduces mechanically evoked neuronal firing but this was only significant for uninjured knee pinch at 60g. (n=2). F) A raw trace from one example ipsilateral WDR neuron from an early phase saline injected sham control, this demonstrates 3 pre-conditioned baseline controls and a reduction in mechanically evoked neuronal firing with concurrent noxious ear and injected knee pinch. Two-way ANOVA with Bonferroni correction. *P<0.05, **P<0.01, ***P<0.001.

Electrophysiological recordings were also taken from WDR neurons contralateral to injection in late phase MIA and sham animals. DNIC was also abolished in WDR neurons found in the contralateral dorsal horn to MIA injection; there was no reduction in neuronal firing with concurrent noxious ear pinch and when the concurrent noxious ear pinch was placed on the uninjured knee (Figure 5.9B). However, when the concurrent noxious knee pinch was placed on the MIA injected knee (now contralateral to the WDR neuron being recorded) there is a small reduction on neuronal firing (Figure 5.9B). The concurrent noxious MIA injected knee pinch produced an 18%, 14%, and 9% reduction in neuronal firing to 8g, 26g, and 60g respectively in contralateral WDR neurons. Meanwhile, in contralateral WDR neurons in late phase saline injected sham controls the concurrent noxious knee pinch placed on the injected knee produced a 46%, 39%, and 30% reduction in mechanically evoked neuronal firing for 8g, 26g, and 60g respectively (Figure 5.9E). A concurrent noxious ear pinch and concurrent noxious pinch placed on the uninjured knee also produced a reduction in contralateral WDR neuronal firing in late phase sham controls (Figure 5.9E). The reductions in mechanically evoked neuronal firing rates in contralateral WDR neurons observed in late phase MIA animals with a concurrent injured knee pinch were to a lesser extent than that observed in early phase MIA animals and sham controls and were not significantly different to pre-conditioned baseline responses. The loss of DNIC in WDR neurons both ipsilateral and contralateral to MIA injection suggests that the alteration that occurs in the endogenous inhibitory system is a global phenomenon.

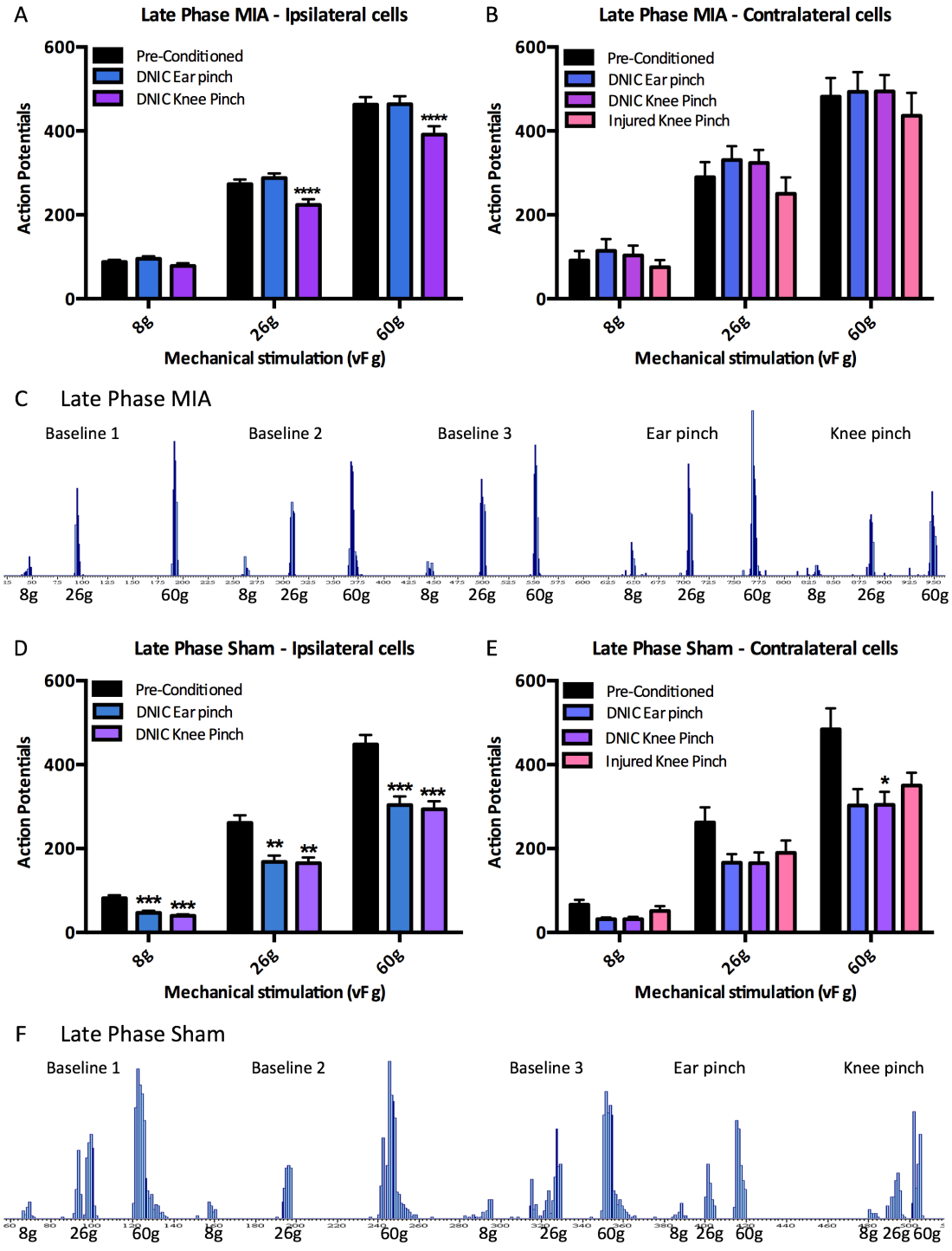


Figure 5.9. The expression of DNIC in late phase MIA animals and saline injected sham controls. A) Electrophysiological recordings of WDR neurons ipsilateral to MIA injection in late phase MIA injected animals. There was no reduction in mechanically evoked neuronal firing with a concurrent noxious ear pinch, however there was a significant reduction when the MIA injured knee was noxiously pinched (n=42). B) There was also no reduction in mechanically evoked neuronal firing in WDR neurons contralateral to MIA injection with a concurrent noxious ear or uninjured knee pinch. Yet there was a reduction in neuronal firing when the MIA injected knee was noxiously pinched but this effect was not significant (n=7). C) A raw trace from one example ipsilateral WDR neuron from a late phase MIA animals, demonstrated three pre-conditioned baseline responses, no reduction in mechanically evoked neuronal firing with concurrent

noxious ear pinch, but a reduction in mechanically evoked neuronal firing with concurrent noxious MIA knee pinch. D) In saline injected sham controls at the same time point as late phase MIA injected animals, there was a significant reduction in mechanically evoked WDR neuronal firing with concurrent noxious ear and ipsilateral knee pinch (n=27). E) Electrophysiological recordings were also taken from WDR neurons contralateral to injection in sham controls. There was a reduction in mechanically evoked neuronal firing with concurrent noxious ear pinch and with a noxious knee pinch on both knees (n=7). F) A raw trace from example WDR neuron in a saline injected sham control, demonstrated three pre-conditioned baseline responses, and a reduction in mechanically evoked neuronal firing with concurrent noxious ear and ipsilateral knee pinch. Two-way ANOVA with Bonferroni post-hoc test. *P<0.05, **P<0.01, ***P<0.001.

The concurrent noxious knee pinches produced varied DNIC responses between ipsilateral and contralateral WDR neurons. For WDR neurons ipsilateral to MIA injection, a concurrent noxious pinch placed on the MIA injected knee (ipsilateral to the cell being recorded) induced a significant reduction in mechanically evoked neuronal firing (Figure 5.9A). Meanwhile, in WDR neurons contralateral to MIA injection a concurrent noxious pinch places on the uninjured knee (ipsilateral to the cell being recorded) did not induce a reduction in mechanically evoked neuronal firing (Figure 5.9B). Yet remarkably when the noxious knee pinch was placed on the MIA injected knee (contralateral to the cell being recorded) it did produce a reduction in mechanically evoked neuronal firing in the contralateral WDR neurons, although this effect was small and not significant (Figure 5.9B). To investigate this further, the concurrent noxious knee pinch was also placed on the uninjured knee (contralateral to the cell being recorded) to assess the DNIC effect in WDR neurons ipsilateral to injection. The concurrent noxious knee pinch placed on the uninjured knee produced a significant reduction in mechanically evoked neuronal firing in early phase MIA animals and both sham groups (Figure 5.10). The reduction in mechanically evoked neuronal firing was around the levels usually observed; in early phase MIA animals the contralateral knee pinch produced a 53%, 38% and 42% reduction in neuronal firing rates to 8g, 26g, and 60g respectively, in early phase sham animals the contralateral knee pinch produced a 46%, 45%, and 38% reduction in neuronal firing rates to 8g, 26g, and 60g respectively, and in late phase sham animals the contralateral late phase sham animals the contralateral knee pinch produced a 47%, 38%, and 37% reduction in neuronal firing rates to 8g, 26g, and 60g respectively (Figure 5.10A, 5.10C and 5.10G). Remarkably, the contralateral noxious knee pinch also produced significant reductions in mechanically evoked neuronal firing in late phase MIA animals, however the inhibitory effects were at much lower levels. In late phase MIA animals the contralateral noxious knee pinch produced only a 12%, 6%, and 7% reduction in neuronal firing rates to 8g, 26g, and 60g respectively (Figure 5.10E and 5.10F). This indicates that although the contralateral

knee pinch produces a reduction in neuronal firing rates in late phase MIA animals, the inhibitory effect is much less powerful than that observed in other animals groups.

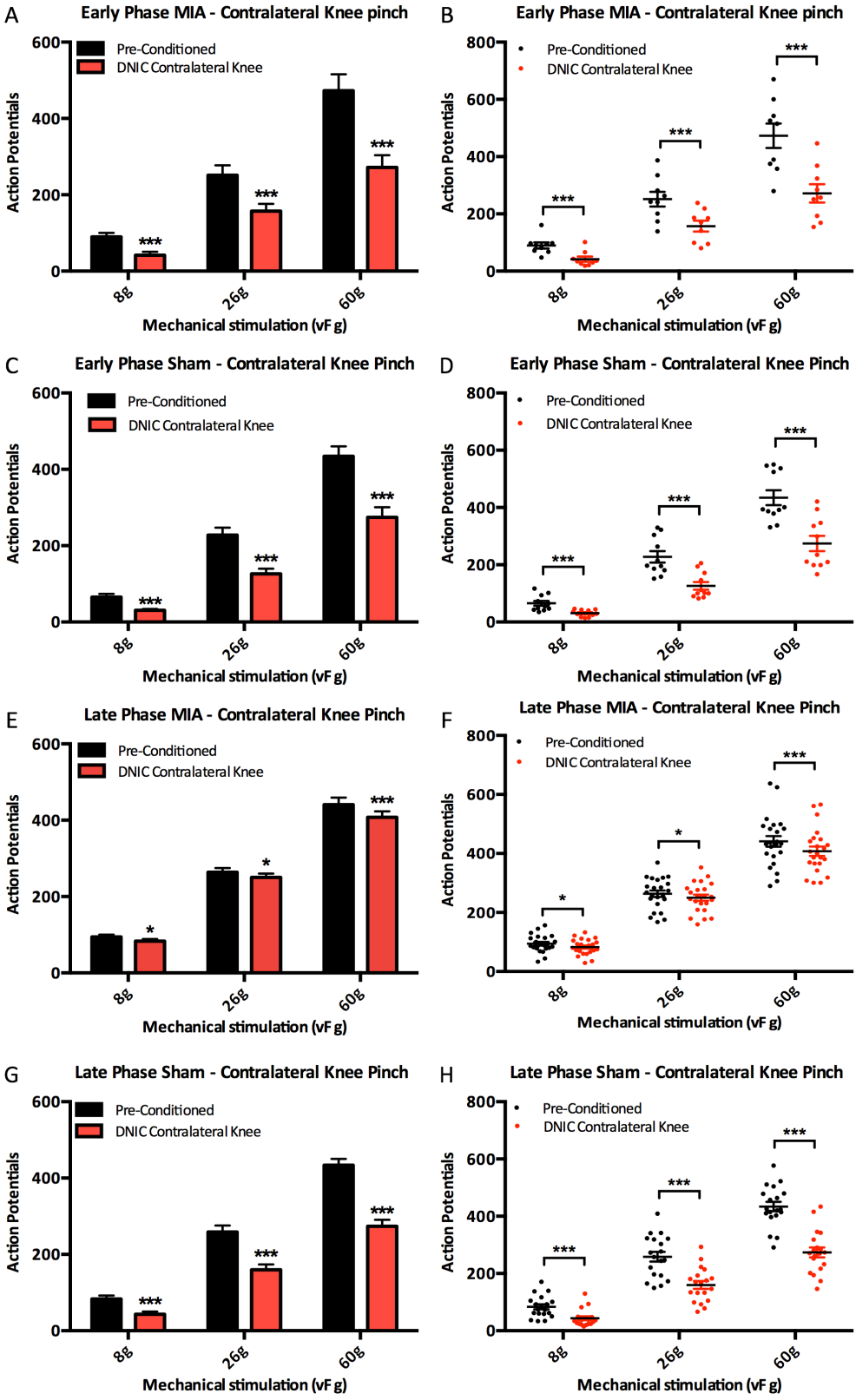


Figure 5.10. The DNIC expression in early and late phase MIA and sham animals in response to a concurrent noxious pinch on the contralateral uninjured knee. A and B) In early phase MIA animals the concurrent noxious contralateral knee pinch produced a significant

reduction in mechanically evoked WDR neuronal firing (n=9). C and D) In early phase saline injected sham controls the concurrent noxious contralateral knee pinch produced a significant reduction in mechanically evoked neuronal firing (n=11). E and F) In late phase MIA injected animals the concurrent noxious contralateral knee pinch produces a much smaller reduction in mechanically evoked neuronal firing rates compared to other groups, yet this effect is still significant (n=23). G and H) In late phase saline injected sham controls the concurrent noxious contralateral knee pinch produced a significant reduction in mechanically evoked WDR neuronal firing rates (n=19). Two-way ANOVA with Bonferroni post-hoc test. *P<0.05, ***P<0.001.

In early phase MIA injected animals, early phase saline injected sham controls, and late phase saline injected sham controls the concurrent noxious ear pinch consistently produced a 30-40% reduction in mechanically evoked neuronal firing (Figure 5.11A, 5.11C, and 5.11E). However, the concurrent noxious ear pinch consistently produced no inhibitory effect on mechanically evoked neuronal firing in late phase MIA injected animals (Figure 5.11A, 5.11C, and 5.11E). Therefore there was significant difference between the percentage neuronal inhibition produced by noxious ear pinch in late phase MIA animals compared to early phase MIA, early phase sham, and late phase sham animals.

A concurrent noxious ipsilateral knee pinch also produced a consistent reduction in WDR neuronal firing at similar levels to that achieved with ear pinch, approximately 30-40%, in early phase MIA, early phase sham, and late phase sham animals (Figure 5.11B, 5.11D, and 5.11F). The level of inhibition on neuronal firing produced by the concurrent noxious ipsilateral knee pinch was much more varied in late phase MIA animals (Figure 5.11B, 5.11D, and 5.11F). The concurrent noxious knee pinch produced a reduction in mechanically evoked WDR neuronal firing in some late phase MIA animals, but this was incapable of producing a DNIC response in other animals within the late phase MIA group. Despite the varied responses observed in late phase MIA animals with concurrent noxious knee pinch, the level of neuronal inhibition produced was to a lesser extent in this group compared to early phase MIA, early phase sham, and late phase sham animals, such that there was a significant difference between the percentage neuronal inhibition produced by noxious ipsilateral knee pinch in late phase MIA animals compared to all other groups.

The similar neuronal inhibition observed in early phase MIA animals and sham controls with both noxious ear and knee pinch suggests early phase MIA animals have a normally functioning DNIC pathway. Meanwhile, the significant differences in neuronal inhibition produced by noxious ear and knee pinch between late phase MIA animals and sham

controls suggests late phase MIA animals have an abolished DNIC system, potentially due to an alteration in the descending controls arising in the brainstem.

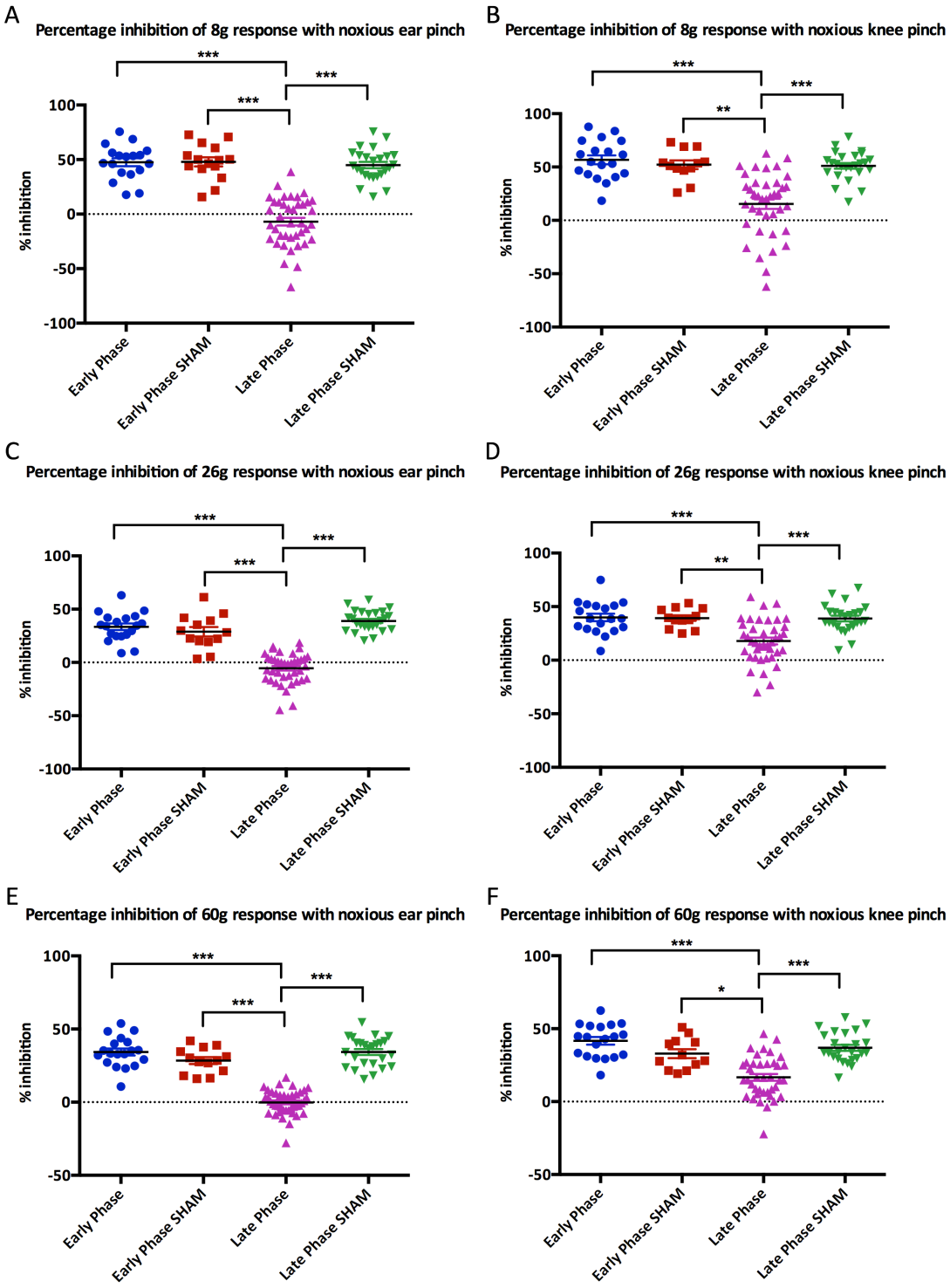


Figure 5.11. The percentage neuronal inhibition produced by concurrent noxious ear and knee pinch in early and late phase MIA and sham animals. A) A concurrent noxious ear pinch produced a consistent inhibition of the 8g mechanically evoked neuronal response in early phase MIA, early phase sham and late phase sham animals, this was significantly different to late phase MIA animals where the concurrent noxious ear pinch did not produce an inhibitory effect on neuronal firing rates. B). A conditioning noxious knee pinch produced a consistent inhibitory

effect on the 8g mechanically evoked neuronal firing rate in early phase MIA, early phase sham and late phase sham animals, while the neuronal inhibition was much more varied in late phase MIA animals and significantly lower than other groups. C) A concurrent noxious ear pinch produced a consistent inhibition of neuronal firing in response to a 26g stimulation in early phase MIA, early phase sham and late phase sham animals, this was significantly different to late phase MIA animals where the concurrent noxious ear pinch did not produce an inhibitory effect on neuronal firing rates. D) A conditioning noxious knee pinch produced a consistent inhibition of 26g mechanically evoked neuronal firing rates in early phase MIA, early phase sham and late phase sham animals, while the neuronal inhibition in late phase MIA animals was much more varied and at a significantly lower level compared to other groups. E) A conditioning noxious ear pinch consistently inhibited 60g mechanically evoked neuronal firing in early phase MIA, early phase sham and late phase sham animals, this was significantly different to late phase MIA animals where the concurrent noxious ear pinch did not produce an inhibitory effect on 60g mechanically evoked neuronal firing rates. F) A concurrent noxious knee pinch produced a consistent inhibition of 60g evoked neuronal firing rates in early phase MIA, early phase sham and late phase sham animals, while the inhibitory effect produced by noxious knee pinch in late phase MIA animals was more varied and at a significantly lower extent. (Early phase MIA n=19, early phase Sham n=13, late phase MIA n=40, late phase Sham n=25). Kruskall-Wallis independent samples one-way ANOVA. *P<0.05, **P<0.01, ***P<0.001.

5.3.6 Microgliosis and astrogliosis in the dorsal horn of MIA animals

The activation of spinal glial cells is thought to contribute to the development of central sensitisation; both inflammatory and neuropathic chronic pain states are associated with the activation of neuroimmune cells (See Section 1.1.6.1)(Calvo and Bennet 2012, Carniglia et al 2017). The MIA model of OA has previously been demonstrated to result in an increased number of activated microglia in the spinal cord at 7, 14 and 28 days post injection (Sagar et al 2011, Thakur et al 2012). Therefore, the contribution of spinal glial cells to the development of central sensitisation was assessed in both early and late phase MIA animals. Specifically, the numbers of microglia and astrocytes in the spinal cord were investigated. The mRNA expression of the microglia marker Ionized calcium binding adaptor molecule 1 (Iba1) and astrocyte marker glial fibrillary acid protein (GFAP) in the lumbar dorsal horn were assessed with quantitative-PCR in early and late phase MIA injected animals and saline injected sham controls. There was an increased level of Iba1 mRNA expression in both MIA groups compared to their respective sham controls especially in late phase animals, indicating microgliosis in the spinal cord of MIA animals, although the effect was not significant (Figure 5.12A and 5.12B). No difference in the levels of GFAP mRNA expression was seen between MIA groups and their respective sham controls, suggesting there is no increase in the number of astrocytes in the dorsal horn following MIA injection at these time points (Figure 5.12A and 5.12C). The rise in the levels of microglia in the dorsal horn of MIA animals, particularly in late phase animals suggests the potential proliferation of microglia, which may be contributing to central changes within the spinal cord and the associated pain-like behaviour in MIA animals.

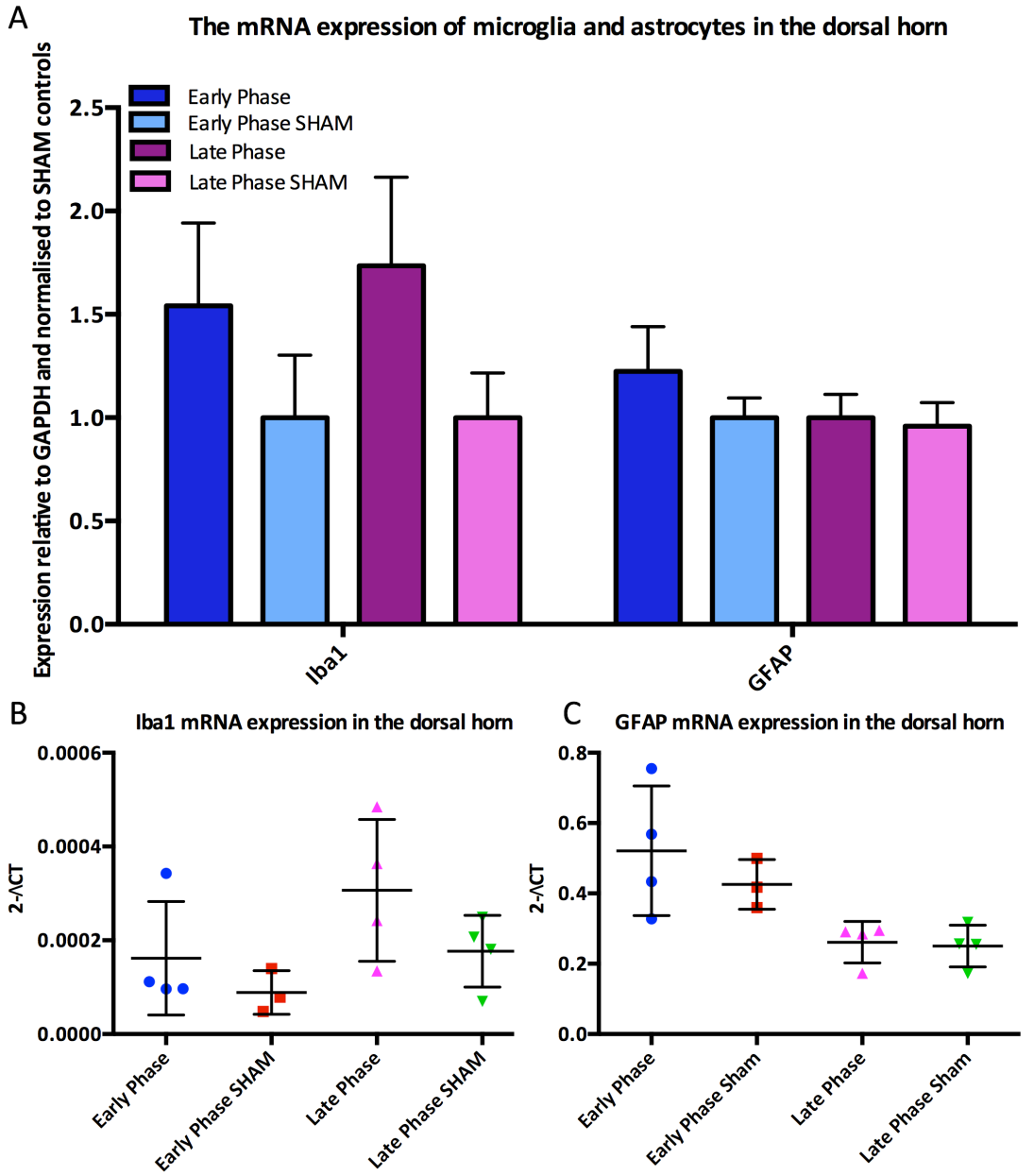


Figure 5.12. The mRNA expression of the microglia marker Iba1 and the astrocyte marker GFAP in the dorsal horn of MIA animals and sham controls. A) The mRNA expression of Iba1 and GFAP in the dorsal horn relative to the housekeeping gene GAPDH and normalized to sham control values. This demonstrates an increase in the levels of Iba1 expression in MIA animals compared to their sham control groups, but no difference in the levels of GFAP expression (EP n=4, EPS n=3, LP n=4, LPS n=4). B) The mRNA expression of Iba1 in the dorsal horn relative to the housekeeping gene GAPDH, this indicates that late phase MIA animals had consistently higher levels of Iba1 mRNA while the Iba1 expression in early phase MIA animals was more varied. C) The mRNA expression of GFAP in the dorsal horn relative to the housekeeping gene GAPDH, this demonstrates no difference in the mRNA expression of GFAP between MIA groups and their respective sham control group. A Kruskall-wallis independent samples one-way ANOVA was used to test for statistical differences between MIA and sham groups when the mRNA expression was normalized to sham. A One-way ANOVA with Bonferroni correction was used to test for statistical differences in mRNA expression represented as 2-ΔCT values between MIA and Sham groups.

5.3.7 The expression of pro-inflammatory cytokines in the MIA model

Activated spinal glia release proinflammatory cytokines into the central nervous system and enhanced levels of key proinflammatory cytokines, including IL-1β, TNFα, and IL6, have previously been demonstrated in the spinal cord following peripheral injury (Zhang and An 2007, Lopes-Castejon and Brough 2011). Additionally, the levels of proinflammatory cytokines are elevated in the synovium, chondrocytes, and subchondral bone in OA patients, and form part of an inflammatory soup that contributes to peripheral sensitisation (See section 1.1.4.1 and 1.3.3). Therefore, the mRNA expression levels of the proinflammatory cytokines, IL-1β, TNFα, and IL6, were investigated both centrally in the dorsal horn and peripherally in DRGs in MIA animals and sham controls.

The mRNA expression of IL-6 and TNFα in the dorsal horn remained relatively constant in both MIA and sham controls groups (Figure 5.13A, 5.13C and 5.13D). However, the mRNA expression of IL-1β was increased in late phase MIA animals compared to their sham control group, although this was not significant (Figure 5.13A and 5.13B). There was also a rise in the mRNA expression of the microglia marker Iba1 in the late phase dorsal horn, as IL-1β expression is enhanced following the activation of microglia these two findings are potentially linked (Zhang and An 2007).

Meanwhile, in the DRGs the mRNA expression of IL-1β and IL-6 remained similar between both MIA groups and their respective sham control groups (Figure 5.13E, 5.13F and 5.13G). When the mRNA expression was normalized to the levels observed in sham, the mRNA expression of TNFα appeared to be increased in the DRGs of late phase MIA animals, but this difference was not significant (Figure 5.13E). However, when the mRNA expression was expressed as the 2-ΔCT values it is clear that only one data point in the late phase MIA group is skewing the data such that the levels appear to be increased, as the remaining late phase MIA expression levels remain similar to the respective late phase sham group (Figure 5.13H).

Overall, these findings indicate there may be a slight increase in the release of the pro-inflammatory cytokine IL-1β in the dorsal horn of late phase MIA animals, potentially due to the activation of microglia, but there is no increase in the levels of IL-6 and TNFα in the dorsal horn of MIA animals. Furthermore, there does not appear to be an increase

in the levels of the pro-inflammatory cytokines IL-1β, TNFα, and IL6, in the periphery at the level of the DRG in either early or late phase MIA animals.

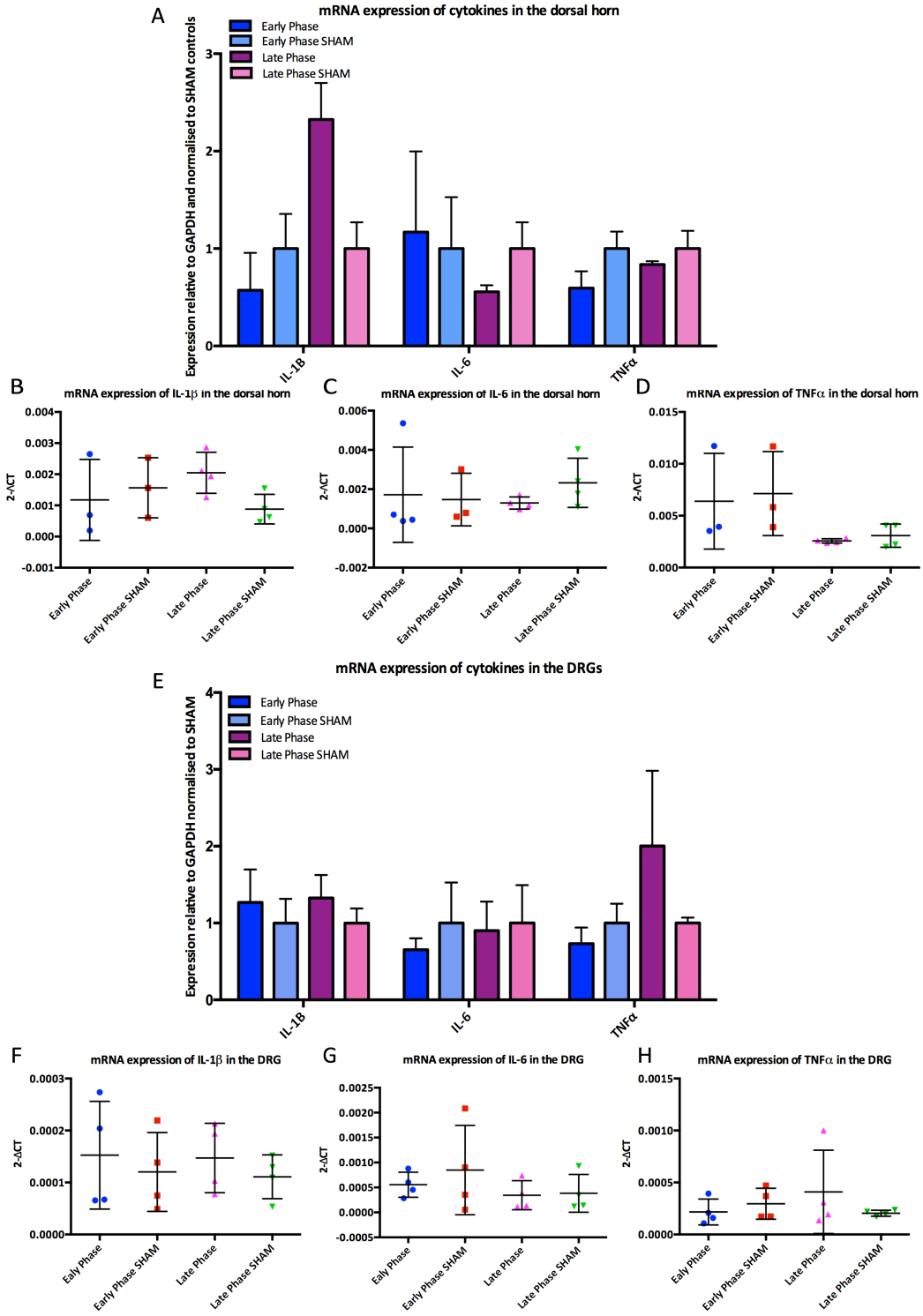


Figure 5.13. The mRNA expression of the pro-inflammtory cytokines IL-1β, IL6, and TNFα, in the dorsal horn and DRGs of early and late phase MIA animals and sham controls. A) The mRNA expression of the proinflammatory cytokines IL-1β, IL6, and TNFα in the dorsal horn of early and late phase MIA animals relative to the housekeeping gene GAPDH and normalized to their respective Sham group. This demonstrates an increase in the mRNA levels of IL-1β in the

dorsal horn of late phase MIA animals (EP n=4, EPS n=3, LP n=4, LPS n=4). B) The mRNA expression of IL-1β in the dorsal horn of early and late phase animals relative to the housekeeping gene GAPDH. This indicates that early phase MIA and early phase Sham animals have similar levels of IL-1β mRNA expression, while late phase MIA animals appear to have slightly increased levels of IL-1β mRNA expression compared to the late phase Sham group but this difference is not significant. C) The mRNA expression of IL-6 in the dorsal horn of early and late phase MIA animals relative to housekeeping gene GAPDH, indicates similar IL-6 mRNA expression levels between both MIA groups and their relative sham controls. D) The mRNA expression of TNFα in the dorsal horn of early and late phase animals relative to housekeeping gene GAPDH, this indicates similar TNFα mRNA expression levels between both MIA groups and their respective sham controls. E) The mRNA expression of the proinflammatory cytokines IL-1β, IL6, and TNFα in the DRGs of early and late phase MIA animals relative to the housekeeping gene GAPDH and normalized to their respective sham group. This demonstrates an increased expression level of TNFα in the DRGs of late phase MIA animals yet this different is not significant. The levels of IL-1β and IL6 remain similar between groups. (EP n=4, EPS n=4, LP n=4, LPS n=4). F) The mRNA expression of IL-1β in the DRGs of early and late phase animals relative to the housekeeping gene GAPDH indicates similar expression levels between groups. G) The mRNA expression of IL-6 in the DRGs of early and late phase MIA animals relative to the housekeeping gene GAPDH, indicates similar levels of expression between groups. H) The mRNA expression of TNFα in the DRGs of early and late phase MIA animals relative to the housekeeping gene GAPDH indicates overall similar levels of expression between groups. A Kruskall-wallis independent samples one-way ANOVA was used to test statistical differences when the expression levels were normalized to the relative sham group. A One-way ANOVA with Bonferroni correction was used to test statistical differences between mRNA expressions expressed as 2-ΔCT values between MIA and Sham groups.

5.4 Discussion

This study examined several pathological features associated with the MIA model of OA at both early (2-6 days post injection) and late (at least 14 days post injection) time points. Firstly, the development of pain like behaviour and the degree of cartilage joint damage were assessed. Secondly, the expression of DNIC was investigated using electrophysiological recordings from WDR neurons in the deep dorsal horn to assess any changes in the descending inhibitory system. Finally, the involvement of spinal glia cells and pro-inflammatory cytokines and their contribution to peripheral and central sensitization were evaluated.

This study indicated that both early and late phase MIA animals develop significant mechanical hypersensitivity on the hind paw and place significantly less weight on the injured limb. Despite the development of pain-like behaviour, early phase animals demonstrate very little cartilage loss within the joint. Meanwhile, knee histology in animals from the late phase group demonstrated severe cartilage loss. The early phase MIA animals displayed a normally functioning DNIC system, as both concurrent noxious ear and knee pinch produced a significant reduction in mechanically evoked neuronal firing at levels similar to that observed in sham controls. However, the concurrent noxious ear pinch produced no inhibition on neuronal firing in late phase animals suggesting an alteration in the descending inhibitory system. Interestingly, DNIC could be induced in some late phase MIA animals by a concurrent noxious pinch placed on the MIA injured knee, which may demonstrate that pinching the injured knee produces a noxious stimulation of adequate strength to trigger descending inhibition. Furthermore, a rise in the levels of microglia and the pro-inflammatory cytokine IL-1β were identified in the ipsilateral dorsal horn of late phase MIA animals, which may contribute to the development of central sensitisation.

5.4.1 The clinical relevance of the MIA model

Although animals models cannot mimic the exact aetiology of OA, a clinically relevant model should develop similar pathological features and symptoms, to better our understanding of the mechanisms involved in the development of disease. As pain is the major cause for patients reporting OA in the clinic, and this thesis focuses on the development of chronic pain, it was important to use an OA model that develops a significant pain phenotype. As the MIA model has previously been reported to develop

both histopathological joint damage and ongoing pain in the joint and at distant sites, this model was selected for the study.

5.4.1.1. The importance of MIA dose

A range of MIA doses, from 0.1mg to 4.8mg, have been demonstrated to dose and time dependently initiate structural joint pathology and pain like behaviour (Guingamp et al 1997, Bove et al 2003, Pomonis et al 2005, Ogbanna et al 2013). Low doses of MIA have been demonstrated to cause low level destruction of the articular cartilage, while high doses produce more structural damage to the joint such as subchondral bone damage (Udo et al 2016). The most commonly used MIA doses for studying the pain associated with OA are 1mg and 2mg (Thakur et al 2012). Both 1mg and 2mg doses of MIA have been demonstrated to result in a significant behavioural profile indicating joint pain and mechanical hypersensitivity of the paw (Thakur et al 2012, Mapp et al 2013). Additionally, both 1mg and 2mg MIA doses cause structural pathology in the knee characteristic of clinical OA, including chondrocyte degeneration, a loss of proteoglycans, and destruction of articular cartilage (Thakur et al 2012, Mapp et al 2013). In fact it has been reported there is no difference in the severity of histopathology between the two doses (Thakur et al 2012). However, a previous thesis produced by the lab reported a lack of involvement from descending serotonergic and noradrenergic pathways influencing spinal cord excitability in the 1mg MIA model (Townson 2017). This contrasts to electrophysiological studies in the 2mg MIA model where an increased serotonergic facilitatory drive and decreased noradrenergic inhibitory drive have been reported to influence WDR neurons (Rahman et al 2009, Burnham and Dickenson 2013). As discussed in Chapter 4, the descending noradrenergic and serotonergic pathways are believed to be crucial for the expression of DNIC. As this thesis aimed to investigate how adaptions due to plasticity in the central nervous system may be contributing to alterations in DNIC and the pain associated with OA, the 2mg MIA model was chosen.

5.4.1.2 Structural pathology in the knee and the associated pain behaviour

The MIA model has faced criticism that while it displays symptoms of joint pain and pain distant to the joint, which may represent referred pain observed in patients, the initiating events and joint pathology following MIA injection are not representative of human OA (Little and Zaki 2012). The behaviour and histology scores in early phase

animals reported in this study agree with this criticism, as significant pain-like behaviour develops in the absence of joint damage. As discussed in Section 5.1.2.1 the MIA injection causes an initial inflammatory infiltrate of monocytes, neutrophils and basophils in the joint, which peaks around day 3 and begins to decline at approximately day 7 (Guzman et al 2003, Clements et al 2009). Interestingly, this study demonstrated bi-phasic pain like behaviour in the MIA model, as the animals reluctance to place weight on the injured limb and mechanical hypersensitivity appears to recover between days 4-7, which is in agreement with previous findings (Bove et al 2003, Thakur et al 2012, Kelly et al 2013). Together these findings indicate that the initial pain behaviour may be due to the general toxicity of the MIA injection and the subsequent inflammatory response. However, it should be considered that that only cartilage damage was scored with histology and since cartilage is both anueral and avascular, damage to cartilage cannot be the direct cause of pain (Hunter et al 2009b). Indeed, there is discordance in patients between the OA associated knee pain and the radiographic knee damage observed (Hannan et al 2000). Therefore, the pain behaviour observed in early phase animals may be a result of damage to other joint structures, such as the synovium. Overall the pain-like behaviour observed in the early phase MIA model may not be a result of joint pathology that is typical to human OA, and therefore may not be entirely representative of the mechanisms driving OA associated pain.

The histopathological joint damage that has common features with human OA, such as cartilage destruction and subchondral bone damage, has been demonstrated to progress in a time-dependent manner following MIA injection (Udo et al 2016). In agreement, this study demonstrated that severe joint damage, including complete loss of articular cartilage and exposure of subchondral bone, occurs in late phase MIA animals. Therefore, it may be concluded that pain behaviour recovers around days 4-7 as the inflammatory infiltrate within the joint declines, and that pain behaviour returns around day 14 due to structural damage within the knee. Indeed, a significant negative correlation was reported between knee histology score and the percentage weight placed on the ipsilateral limb. The relationship between an increase of cartilage damage and the reluctance of the late phase MIA animals to place weight on the injured joint indicates that the pain behaviour observed is due to histopathology in the knee. Overall, this study suggests that the development of pain in late phase MIA animals is due to pathological events within the joint that are similar to clinical features observed in human OA.

5.4.1.3 Time dependency of the MIA model

While the extent of MIA-induced joint pathology has been demonstrated to depend upon MIA dose, it also depends on the time post injection (Guzman et al 2003, Udo et al 2016). While inflammatory changes in the synovium and infrapatellar fat pad have been demonstrated to occur rapidly (less than 7 days post injection), structural joint changes have been demonstrated to occur at later time points (Dunham et al 1992, Clements et al 2009). When 1mg MIA-induced histopathological features were monitored for an extended period of time, it was demonstrated that subchondral bone marrow lesions began to form around day 14, followed by a complete loss of articular cartilage and subsequent fragmentation of subchondral bone at day 28-30, and significant bone remodeling at day 56 (Guingamp et al 1997, Guzman et al 2003). Similarly, imaging studies in OA patients have identified bone marrow lesions, subchondral bone fibrosis and extensive bone remodeling as common clinical features (Wieland 2005, Conaghan et al 2006). Therefore, the histology results in this study and previous studies suggest that at later time points post MIA injection, the more accurately the model may represent structural joint changes and associated joint pain that occur in human OA.

5.4.2 Diffuse Noxious Inhibitory Controls in the MIA model

This study reported that early phase MIA animals have a normally functioning DNIC as both concurrent noxious ear and knee pinch produced a significant reduction in mechanically evoked neuronal firing at similar levels to that observed in saline injected sham controls. This thesis and previous studies have demonstrated that DNIC expression is reliant upon a functioning noradrenergic descending inhibitory system (Bannister et al 2015, Bannister et al 2017). A previous 2mg MIA study reported that early phase MIA animals had a functioning noradrenergic system, as the spinally applied α_2-adrenergic receptor antagonist atipamezole fully reversed the inhibitory effects produced by the SNRI Milnacipran (Burnham and Dickenson 2013). Furthermore, NSAIDs have been demonstrated to be effective in early phase MIA animals, while late phase MIA animals do not respond indicating the development of central sensitisation (Fernihough et al 2004). Together these findings suggest that the pain behaviour demonstrated in early phase animals may be a result of peripheral sensitisation, and the fully functioning DNIC system may indicate a lack of central changes.

On the other hand, this study demonstrated there was a loss of DNIC in late phase MIA animals, as the concurrent noxious ear pinch no longer produced a reduction in

mechanically evoked neuronal firing. It has previously been reported that there is a reduction in descending noradrenergic inhibitory controls in late phase MIA animals, as atipamezole could not longer fully reverse the inhibitory effects of the SNRI milnacipran (Burnham and Dickenson 2013). Additionally, an enhanced descending serotonergic facilitatory drive acting at 5-HT_3 receptors in the spinal cord has been demonstrated in late phase 2mg MIA animals (Rahman et al 2009). Interestingly, in a previous study where DNIC was lost in SNL animals, the spinal application of the 5-HT_3 receptor antagonist ondansetron restored DNIC, indicating that an increased serotonergic facilitatory drive may be contributing to the loss of DNIC (Bannister et al 2015). Furthermore, NSAIDs have been demonstrated to be ineffective at relieving hyperalgesia and allodynia in late phase MIA animals, potentially indicating central changes (Fernihough et al 2004). Therefore, the loss of DNIC in late phase MIA animals observed in this study may be a result of an imbalance in descending controls, specifically a reduced descending inhibitory noradrenergic and enhanced descending facilitatory serotonergic system.

Remarkably, in some late phase MIA animals a reduction in neuronal firing was observed when the concurrent noxious pinch was placed on the injured knee. Firstly, this indicates that the DNIC system is not completely abolished and proposes that activation of the reduced descending noradrenergic system by the conditioning stimulus is no longer sufficient to override the enhanced descending serotonergic faciltatory drive acting at the spinal cord. Overall, the imbalance in descending inhibitory and serotonergic controls is masking the expression of DNIC, this agrees with previous studies demonstrating DNIC can be revealed pharmacologically (Bannister et al 2015, Bannister et al 2017). Previous studies using electrophysiological recordings from joint afferents have demonstrated MIA produces a graded sensitisation and increased firing rate (Schuelert and McDougall 2009, Schuelert and McDougall 2012). Furthermore, a Nav1.8 channel blocker reduced the firing rate of joint afferents in response to noxious rotation of the joint but had no effect during non-noxious rotation (Schuelert and McDougall 2012). This may indicate that upon noxious conditioning stimulation of the MIA injured knee there is likely an increased firing rate of joint afferents and an increased transduction of nocicieptive signal via sodium channels to the dorsal horn. Therefore, the concurrent MIA injured knee pinch may now produce a sufficient nociceptive signal to activate descending inhibitory controls and produce a small level of neuronal inhibition.

Surprisingly, the concurrent contralateral uninjured knee pinch also appeared to produce a significant reduction in neuronal firing in late phase animals, but this was at much lower levels than that observed in early phase animals and sham controls. As discussed in Section 2.1.3, a 10% variation in neuronal firing is expected. Therefore, the marginal reduction in number of action potentials may be due simply to variations in neuronal firing, which may be significant due to large n numbers. Due to the low levels of neuronal inhibition observed with concurrent contralateral knee pinch in late phase MIA animals of 12%, 6%, and 7% for 8g, 26g and 60g respectively, I would be reluctant to characterize this as a DNIC response.

5.4.3. The involvement of spinal glia in the MIA model

As discussed in Section 1.1.6.1, the glial cells form a huge part of the central nervous system, can become activated in response to peripheral tissue damage, and release factors that interact with neurons to regulate their function (McMahon and Malcangio 2009, Clark and Malcangio 2012). In particular, immunohistochemistry studies have demonstrated the proliferation and activation of microglia in the L3, L4 and L5 levels of the ipsilateral spinal cord following MIA injection (Sagar et al 2011, Thakur et al 2012, Ogbonna et al 2013). This study found that there was a rise in the mRNA expression of Iba1, a microglia specific calcium binding protein, in late phase MIA animals compared to late phase sham controls but this effect did not reach significance. While measuring mRNA levels of Iba1 provides information on the proliferation of microglia, it cannot identify their activation. Indeed, a previous study demonstrated that the increase in Iba-1 expressing microglia in the ipsilateral dorsal horn following MIA injection did not reach significance at day 7 but did at day 28 (Ogbonna et al 2013). Therefore, at our time point of day 14 there may be considerable activation of microglia as previously described but a less substantial proliferation (Sagar et al 2011, Thakur et al 2012, Ogbonna et al 2013).

One mechanism in which activated microglia can modulate the function of spinal neurons is through the release of pro-inflammatory cytokines, particularly IL-1β, IL-6 and TNFα (Schomberg and Olsen 2012, Calvo and Bennett 2012). This study found a rise in IL-1β mRNA expression in the spinal cord of late phase MIA animals compared to late phase sham controls, but this effect did not reach significance. It should be noted that this study used the entire lumbar dorsal horn for qPCR, while microgliosis has previously been described specifically in L3, L4 and L5 lumbar levels, therefore the increased levels of microglia and subsequent release of pro-inflammatory cytokines in

late phase MIA animals may be diluted in these results. IL-1β has been shown to enhance AMPA and NMDA induced currents and suppress GABA and glycine induced currents in the dorsal horn (Kawaski et al 2008). Overall, this rise in mRNA expression could indicate that the peripheral tissue damage caused by the MIA injection in late phase animals is resulting in microgliosis in the ipsilateral dorsal horn and the subsequent release of the pro-inflammatory cytokine IL-1β. Microgliosis and the release of IL-1β may contribute to an increased excitatory and decreased inhibitory synaptic transmission at second order neurons in the dorsal horn, contributing to the development of central sensitisation (Kawaski et al 2008).

Astrocytes are the most abundant glial cell in the central nervous system, and they regulate the synaptic levels of K^+ and glutamate (Milligan and Watkins 2009, Bradesi 2010). This study found no difference in the mRNA expression levels of GFAP between either early or late phase MIA animals and their respective sham control group. This is in agreement with previous studies that identified no increased in spinal GFAP immunofluorescence at days 7 and 14 post MIA injection (Sagar et al 2011, Thakur et al 2012, Ogbonna et al 2013). However, there are conflicting findings for GFAP immunofluorescence at later time points, with one study identifying a significant increase and another finding no alteration in GFAP intensity at 28 days post MIA injection (Sagar et al 2011, Ogbonna et al 2013). GFAP is not be an absolute biomarker of astrocytes as it may not detect non-reactive astrocytes, but it is a reliable and sensitive biomarker for reactive astrocytes (Sofroniew and Vinters 2010). Therefore, the results from this study and previous findings suggest astrogliosis is not contributing to the development of central sensitisation in MIA animals at these time points.

5.4.4 Peripheral pro-inflammatory cytokines in the MIA model

As discussed in Section 1.3.3, there are increased levels of pro-inflammatory cytokines, particularly IL-1β and TNFα in several OA joint structures, which mediate the progression of cartilage damage and joint destruction (Kapoor et al 2011). IL-1β and TNFα can perpetuate the OA disease state in the joint as they result in an inhibition of anabolic activities in the chondrocytes, and subsequently prevent the synthesis of ECM components (Goldring 1994, Kapoor et al 2011). The mRNA levels of the proinflammatory cytokines, IL-1β, TNFα, and Il-6 were assessed at the level of the DRG to investigate any inflammatory response in peripheral neurons. This study identified no alteration in the mRNA levels of any of these pro-inflammatory cytokines at the level of the DRG in either early or late phase MIA animals. This may indicate that any

neuronal sensitisation at the level of the DRG in MIA animals is not due to an inflammatory infiltrate, but it could also be that the pro-inflammatory cytokines are being produced and secreted at the level of the joint, and as such the measurement of mRNA levels at the DRG would not detect this. It has previously been demonstrated that there are elevated levels of the pro-inflammatory cytokines TNFα and IL-6 in knee soft tissue structures following MIA injection (Orita et al 2011). Therefore, analysing the expression of pro-inflammatory cytokines in the joint may have been a better approach to better understand the local inflammation that occurs in the MIA model.

5.4.5 Concluding remarks

This study indicates that while both early and late phase MIA animals develop significant pain behaviour, late phase MIA animals also have severe cartilage damage and so may be the more accurate representation of clinical OA. The synaptic plasticity that exists in the dorsal horn means that after 14 days of receiving a barrage of nociceptive information from the MIA injured knee it is likely central sensitisation develops, and this potentially occurs through microgliosis and the subsequent release of pro-inflammatory cytokines, which modulate excitatory and inhibitory currents. The loss of DNIC in late phase MIA animals indicates an imbalance in descending facilitatory and inhibitory controls from the brainstem. However, a concurrent noxious pinch on the MIA injured knee produces a reduction in neuronal firing, potentially suggesting that noxiously stimulating the sensitised joint afferents and increasing the transduction of the nociceptive signal to the dorsal horn is sufficient to produce inhibitory control. Overall, early phase animals may respond to analgesics that act peripherally, such as NSAIDs or Nav1.8 channel blockers. Whereas, therapeutics that target the central nervous system or act to restore the balance in descending systems from the brainstem may be the most effective option for relieving pain in late phase MIA animals and also in a subset of OA patients.

6. Pharmacologically restoring Diffuse Noxious Inhibitory Controls and investigating a neuropathic component in the monoiodoacetate model of Osteoarthritis.

6.1 Introduction

As discussed in Section 1.3.5 there are several analgesics used in the clinic to treat the pain associated with OA, with most of these treatment options aiming to relieve inflammation or replace lost ECM components in the joint (Wieland et al 2005). Yet, many OA patients develop referred pain and a subset suffers with chronic pain even following total knee replacement surgery, which suggests discordance between nociceptor activation and the resulting pain experience (Malfait and Schnitzer 2014). Together these features indicate that the pain associated with OA cannot always be considered purely peripheral, but there are fewer treatment options available which target the central nervous system to tackle centrally driven chronic pain (Gwylim et al 2009, Wylde et al 2011). Additionally, a subset of OA patients report neuropathic features to their pain and so may benefit from analgesics tailored to treat the neuropathic component (Thakur et al 2014). Overall, the advancement in the understanding of the peripheral and central molecular mechanisms that cause the pain associated with OA and the shift towards personalized medicine in the clinic, means patients are now being more thoroughly diagnosed to understand their individual pain phenotype in order to prescribe the most effective analgesic.

6.1.1 Spinal and supraspinal plasticity in Osteoarthritis patients

As discussed in Section 1.3.6 and Chapter 5, the continuous barrage of nociceptive information from the joint and the high level of plasticity that exists in the CNS, often results in hypersensitivity of second order neurons in the dorsal horn and the subsequent development of central sensitisation. The exact molecular mechanisms behind the development of central sensitisation are discussed in Section 1.1.6, but overall a facilitation in synaptic neurotransmission, an increase in membrane excitability of second order neurons, and a lack of inhibitory control from interneurons are the main drivers (Malfait and Schnitzer 2014). Additionally, peripheral tissue

damage can activate spinal glia cells, which subsequently modulate neuronal function (McMahon and Malcangio 2009). The result is a reduction of activation threshold, increased spontaneous activity, and an expansion of the receptive field in dorsal horn neurons, which is presented in the clinic as allodynia, hyperalgesia and referred pain at sites distant to initial joint damage (Malfait and Schnitzer 2014). The development of central sensitisation may explain the discordance between radiological joint damage and the pain experienced by OA patients (Hannan et al 2000).

In addition, spinal cord neurons receiving input from the joint are under the influence of descending controls arising in the brainstem, therefore impairment of descending controls may also contribute to an exaggerated pain response in OA patients. Indeed, a study investigating sensitization in patients with painful knee OA demonstrated reduced pressure pain thresholds and facilitated temporal summation to repeated pressure pain stimulation at the both the knee and sites distant to the joint, which reflects altered spinal processing of afferent input (Arendt-Nielsen et al 2010). In addition, these patients had a reduced CPM response, indicated the importance of central sensitisation and the impairment of descending inhibition in contributing to the OA associated chronic pain (Arendt-Nielsen et al 2010). Similarly, a study in patients with painful hip OA reported that pressure pain thresholds could not be modulated by heterotopic noxious conditioning stimulation, whereas this produced an increased threshold in controls, indicating a dysfunction DNIC system (Kosek and Orderberg 2000). However, when these patients were re-assessed in a pain free state 6-14 months after total joint replacement surgery, pressure pain thresholds could be modulated by heterotopic noxious conditioning stimulation, indicating the return of a functional DNIC system (Kosek and Orderberg 2000). This finding agrees with previous studies that report the DNIC system is not completely abolished in chronic pain states, rather the enhanced descending faciliatory system overrides the reduced descending inhibitory system activated by the conditioning stimulus (Bannister et al 2015, Bannister et al 2017). It has previously been demonstrated that DNIC can be restored pharmacologically, but this study highlights that simply treating the peripheral nociceptive drive could also restore DNIC. However, this finding contradicts with the observation that a subset of patients remain with chronic pain following total joint replacement surgery (Kosek and Orderberg 2000, Wylde et al 2011). Overall, these findings highlight the importance of considering spinal hypersensitivity and dysfunctional descending controls when tackling the clinical manifestations associated with chronic OA pain.

The chronic pain associated with OA may also be influenced by modulation at the supraspinal level; morphological alterations and increased levels of activity have been reported in brain regions in OA patients with signs of central sensitisation (Gwilym et al 2009, Rodriguez-Raecke et al 2009, Parks et al 2012). Firstly, Gwilym and colleagues carried out an fRMI study in patients with chronic hip OA; they identified increased activity in the PAG midbrain area when stimulating areas of referred pain, indicating PAG activity may be influencing the development of central sensitisation (Gwilym et al 2009). Additionally, Rogriguez-Raecke and colleagues monitored whole brain structures in patients with chronic hip OA and found a decrease in grey matter in the anterior cingulate cortex (ACC), dorsolateral prefrontal cortex (dlPFC), amygdala and brainstem compared to age-matched controls (Rodriguez-Raecke et al 2009). Remarkably, when this procedure was repeated in a subset of these patients who were pain free following total joint replacement an increase in grey matter in the ACC, dlPFC, amygdala and grey matter was observed, indicating the grey matter abnormalities were reversible consequences of the nociceptive drive from the injured hip (Rodriguez-Raecke et al 2009). Finally, a further fMRI study in patients with painful knee OA found a dissociation between stimulus evoked and spontaneous pain related brain area activity, with the latter associated with the activation of prefrontal-limbic regions (Parks et al 2012). Interestingly, the brain areas highlighted that display morphological or activity-related changes, have all been reported to play a role in the DNIC response (See Section 1.2.4), further indicating the important role played by this system towards the development of chronic OA pain.

While the DNIC system represents one descending pathway controlled by the SRD, a further descending system in the dorsolateral funiculus (DLF) exists, which is controlled from other brainstem regions such as the RVM (Bannister and Dickenson 2016a). As discussed in Section 1.1.5.5, the RVM sends modulatory projections directly to the spinal cord, and can exert bi-directional control of the pain response through the activation of pronociceptive ON-cells or antinociceptive OFF-cells (Heinricher et al 1994). As the RVM receives direct inputs from the PAG, PbN, and LC, and is considered the final relay for descending modulation to the spinal cord, this descending pathway may also be contributing to the development of central sensitisation (Ossipov et al 2010, Ossipov et al 2014). In addition to the interaction between the RVM and the noradrenergic nucleus, the LC, the activation of RVM descending projections results in the release of serotonin in the spinal cord (Ossipov et al 2014). As DNIC has been demonstrated to rely upon noradrenergic and serotonergic neurotransmission, this may suggest a complex

interplay between the two descending systems is required for the final DNIC response (Bannister and Dickenson 2016a). Furthermore, an fMRI study in human subjects demonstrated that increased pain perception due to central sensitisation is associated with activation of this brainstem region (Lee et al 2008). Overall, neuronal plasticity in supraspinal brain structures and alterations in both ascending and descending systems should be considered when tackling the development and maintenance of central sensitisation in OA patients.

Experimentally probing the DNIC response in animals models holds great clinical relevance, and allows us to assess the functionality of descending controls in chronic pain states (Edwards et 2003). The lack of DNIC observed in the late phase MIA model (See Chapter 5), indicates that neuronal plasticity within the spinal cord, supraspinal brain structures, and descending controls may be modulating the pain response in this model. Therefore, through pharmacologically manipulating descending controls and probing the DNIC response in the MIA model, we can better understand the mechanisms behind its loss and better explore therapies that restore this system.

6.1.2 Tapentadol: manipulating the descending noradrenergic and opioidergic systems to achieve analgesia

Tapentadol Hydrochloride ((-)-(1R,2R)-3-(3-dimethylamino-1-ethyl-2-methyl-propyl)-phenol hydrochloride) is a centrally acting novel analgesic that combines two modes of action in one molecule (Wade and Spruill 2009, Tzschentke et al 2009). Tapentadol is a μ-opioid receptor (MOR) agonist and hence activates descending opiodergic controls, while also functioning as a NRI, which functions to increase the synaptic availability of noradrenaline and subsequently produces analgesia through the activation of α_2-adrenoceptors (Schroder et al 2011, Tzschentke et al 2012). Both the opioid antagonist naloxone and the α_2-adrenoceptor antagonist atipamezole partially block tapentadols analgesic effect, and whole-cell patch clamping has demonstrated that tapentadol has similar potency for MOR activation and noradrenaline transporter inhibition, indicating the importance of the two distinct modes of actions in mediating analgesia (Schroder et al 2011, Sadeghi et al 2015). Tapentadol has 44-fold lower affinity for the MOR than morphine and yet its analgesic potency is only 2-3 fold lower (Tzschentke et al 2007, Tzschentke et al 2012). In addition, tapentadols NRI activity is moderate, as it has weaker binding affinity for the NA transporter than other NRIs inclusing duloxetine and nisoxetine (Tzschentke et al 2007). The potent analgesic potential of tapentadol, despite

its moderate affinities for the MOR and NA transporter, suggests a synergistic interaction between these two distinguished mechanisms (Schroder et al 2011). Overall, tapentadol has the potential to be a powerful analgesic molecule, which also avoids the adverse side effects associated with opioid analgesia (Tzschentke et al 2014).

6.1.2.1 The efficacy of Tapentadol in patients

Tapentadol has proved an effective analgesic in patients with several chronic pain states in both Phase II and Phase III clinical trials (Wade and Spruill 2009). Phase II clinical trials have investigated patients with moderate to severe chronic pain following bunionectomy or dental surgical extraction, and its efficacy and tolerability compared to morphine, ibuprofen and placebo (Lange et al 2006, Stegmann et al 2006). Both studies indicated that tapentadol provided rapid-onset analgesia and produced a lower incidence of adverse side effects, such as nausea, vomiting and dizziness, than morphine (Lange et al 2006, Stegmann et al 2006). Furthermore, Phase III clinical trials investigated the efficacy and tolerability of tapentadol in comparison to an active control, the opioid oxycodone, in patients with low back pain and chronic pain associated with OA of the hip or knee (Hale et al 2009, Hatrick et al 2009). While both trials indicated that tapentadol provided pain relief at a similar level to oxycodone, they found that tapentadol was associated with an improved gastrointestinal tolerability as fewer patients suffered from adverse effects (Hale et al 2009, Hatrick et al 2009). Overall, these clinical findings suggest tapentadol has the potential to be an effective and well-tolerated analgesic for use in patients with OA associated chronic pain (Wade and Spruill 2009).

6.1.2.2 The efficacy of Tapentadol in animal models

The analgesic efficacy of tapentadol has also been explored in animal models of nociceptive, inflammatory and neuropathic pain (Schroder et al 2010, Bee et al 2011, Schiene et al 2011). Firstly, tapentadol proved to have effective antinociceptive and antihyperalgesic properties in CFA, formalin, and carrageenan-induced models of acute inflammation, and in a CFA-induced arthritis model of chronic inflammatory pain (Schiene et al 2011). Interestingly, the antinociceptive effects of tapentadol in naïve rats were demonstrated to be mediated predominantly through its action at the MOR, while the antihypersensitive effects of tapentadol in a rat SNL model were demonstrated to rely mainly upon the inhibitory actions at the noradrenaline transporter, indicating the precise molecular mechanisms through which tapentadol functions depends upon the particular pain state (Schroder et al 2010). Similarly, Bee and colleagues investigated

the efficacy of tapentadol, in combination with the α_2-adrenoceptor antagonist atipamezole and the opioid receptor antagonist naloxone, in SNL and sham rats (Bee et al 2011). The authors demonstrated both atipamezole and naloxone produced near complete reversal of tapentadol induced inhibitory actions in SNL animals, yet in sham controls tapentadol induced inhibitory actions functioned predominantly through a opioid inhibitory mechanisms (Bee et al 2011). Therefore, the authors proposed a shift in tapentadols analgesic properties from a predominant opiodergic mechanism to a predominant noradrenergic mechanism in neuropathic pain states (Bee et al 2011). Furthermore, the action of the serotonin receptor antagonist ritanserin was explored in combination with tapentadol in both inflammatory and neuropathic animal models (Schroder et al 2010, Schiene et al 2011). Ritanserin proved ineffective at blocking the analgesic actions of tapentadol in both a neuropathic rat model and a CFA-induced arthritis model of chronic inflammations, indicating tapentadol's inhibitory actions do not rely on the serotonin reuptake inhibition (Schroder et al 2010, Schiene et al 2011). Overall, these findings incidate that both MOR agonism and NRI mechansims are crucial for the analgesic actions of tapentadol, with the predominant mechanism depending upon the pain state. Previous reports have suggested that the DNIC system is partly opioid, while this thesis and previous work have demonstrated functional descending noradrenergic controls are crucial for DNIC (Le Bars et al 1981, Bannister et al 2015, Bannister et al 2017). Therefore, the effect of tapentadol on restoring the expression of DNIC is investigated in the late phase MIA model in this study.

6.1.3 A neuropathic component to Osteoarthritis

IASP define neuropathic pain as "pain caused by a lesion or disease of the somatosensory system", which can be further subdivided into central and peripheral neuropathy. Therefore, to assess if a patient is suffering with neuropathic pain, a number of diagnostic criteria and clinical features are examined to determine the likelihood of the pain arising as a direct consequence of damage to the somatosensory system (Treede et al 2008)(Figure 6.1). Conclusively diagnosing chronic pain as a result of neuropathy remains complicated as the symptoms and signs of neuropathy differ extensively between individuals (Suzuki and Dickenson 2000). Firstly, lesions to peripheral nerves can cause a partial or complete loss of afferent inputs to the central nervous system and a subsequent sensory deficit, which demonstrate negative symptoms such as areas of numbness or a loss of sensitivity (Suzuki and Dickenson 2000, Jensen and Baron 2003). Paradoxically, patients often develop peripheral areas of

hypersensitivity adjacent to the areas of sensory loss, which can be identified with positive symptoms such as allodynia and hyperalgesia (Suzuki and Dickenson 2000, Jensen and Baron 2003). Patients with neuropathy can present with just one of these symptoms, while many patients present with multiple combinations of positive and negative symptoms, indicating neuropathic pain can arise from multiple mechanisms (Suzuki and Dickenson 2000). Through thoroughly evaluating the pathology behind the development of the chronic pain, and identifying a potential neuropathic component, practitioners would be better placed to prescribe effective analgesics.

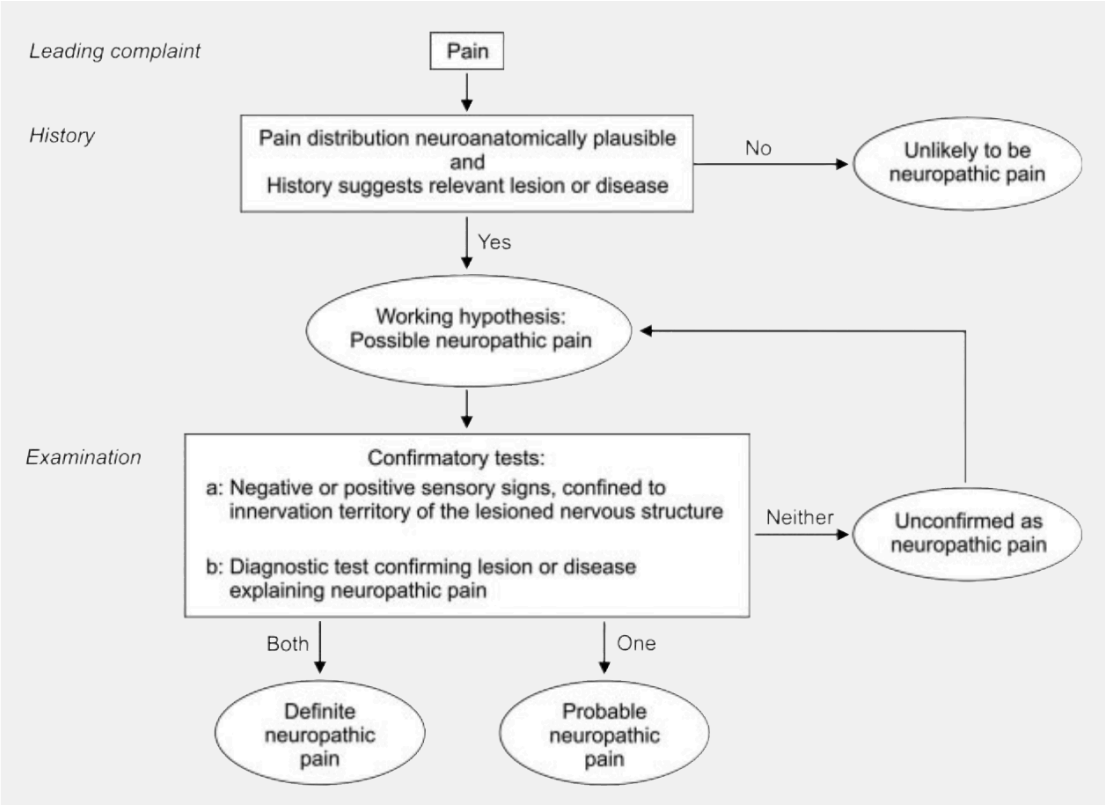


Figure 6.1. A flow chart of the clinical criteria used for diagnosing neuropathic pain. Diagnosing neuropathic pain is a complex task due to the multiple and varied symptoms presented by patients, this flow-chart indicates a tool for diagnosing the probability of neuropathic pain. (Taken from Treede et al 2008).

6.1.3.1 Osteoarthritis patients presenting with neuropathic features

Several studies have reported that subsets of OA patients develop neuropathic features to their pain, indicating an unmet need for therapies that aim to tackle the neuropathic component (Thakur et al 2014). Indications of OA patients experiencing neuropathic pain include patients presenting with hypersensitivity in areas distant to the injured joint, and patients describing pain as shooting or pins and needles with a burning quality (Hawker et al 2008, Wylde et al 2012). Patients with neuropathic pain report a

more severe pain phenotype and a poorer quality of life, hence it is important to provide the appropriate analgesic (Freynhagen et al 2006).

The pain-DETECT questionnaire was originally designed for patients with lower back pain, as a screening tool to identify neuropathic pain components (Freynhagen et al 2006). This questionnaire proved to be a reliable, sensitive and accurate tool for detecting neuropathy in patients with lower back pain, and has now been extended to other chronic pain states (Freynhagen et al 2006). In a cohort of patients with hip OA, those with higher pain-DETECT scores experienced lower pain thresholds for punctate stimuli in areas of referred pain and displayed increased activity in the PAG with fMRI imaging, indicating central sensitisation and supraspinal involvement was correlated with patients with neuropathic components of pain (Gwilym et al 2009). Additionally, a cohort of 259 patients with chronic OA of the knee completed the pain-DETECT questionnaire, and this indicated that a quarter of the patients in this study had neuropathic symptoms localized to the knee (Hochman et al 2011). In a further cohort of patients with chronic knee OA, a higher pain-DETECT score was positively correlated with signs of central sensitisation, assessed through quantitative sensory testing (QST) (Hochman et al 2013). Taken together, these findings indicate that OA patients describing neuropathic features are likely to have developed central hypersensitivity, and may benefit from a more thorough evaluation of their pathology and neuropathy targeted analgesics (Hochman et al 2010).

6.1.3.2 The innervation of the knee: an anatomical case for neuropathy?

The development of neuropathy in OA patients is likely due to compression or damage of peripheral afferent fibres either innervating or surrounding the joint (Suzuki and Dickenson 2000, Thakur et al 2014). As discussed in Section 1.3.4, the articular cartilage is not innervated so cartilage damage cannot be the direct source of pain, and also cannot be the direct cause of neuropathy. However, as all other joint structures contain free nerve endings, the source of neuropathy may be the result of OA induced damaged to such structures (Schaible et al 2009). Indeed, the subchondral bone is densely innervated with small diameter nociceptors and increased incidence of OA pain is associated with bone marrow lesions (Wieland et al 2005, Zhang et al 2011). This may indicate that in extreme late-stage cases of OA with completely degraded articular cartilage and subchondral bone remodeling, extensive nerve damage also occurs. However, MRI scans do not have the strength to identify nerve lesions and confirm neuropathy. In addition, the synovium has a vast supply of unmyelinated C fibres and

undergoes a large inflammatory infiltrate following the initiation of OA (Buchanan and Kean 2002, Sellam and Berenbaum 2010). Therefore, the synovium may be a further source of peripheral nerve lesions, potentially due to inflammatory damage. Given that several innervated joint structures are damaged during OA, there is a feasible anatomical case for the compression or lesion of peripheral nerves within the joint.

The pain associated with OA of the knee is most common within medial and lateral compartments of the joint, in muscles surrounding the joint, and along the upper tibia (Kean et al 2004). The presence of referred pain in sites distant to the injured knee, most often the hip and lower back, is a symptom considered to be associated with neuropathy (Hochman et al 2011). Furthermore, when the pain experience was examined in a cohort of patient with knee and hip OA, many participants used pain descriptors associated with neuropathy, such as burning or tingling (Hawker et al 2008). These neuropathic features may be a result of spinal hyperexcitability, as the accumulation of nociceptive input from multiple joints or surrounding areas may contribute to the development of central sensitisation (Hochman et al 2011). Alternatively, the anatomical distribution of referred pain in joints and surrounding structures observed in knee OA may be reflect the convergence of spinal neurons with receptive fields in the joint and surrounding tissues. Overall, if a patient has a history of multiple joint pain or reports with neuropathic features, the pathology of knee pain should be examined extensively.

6.1.3.3 A neuropathic component to the MIA model

As the extent of joint damage in the MIA model is dependent upon dose and timing, there have been reports that while the 2mg model causes joint degeneration it also evokes neuropathy within and surrounding the joint (Thakur et al 2012). While the expression of inflammatory cytokines in joints tissues and DRGs has been reported to peak around day 7-14 and then decline in the MIA model, the nerve stress marker, activating transcription factor-3 (ATF-3), has been reported to become expressed 7 days following injection and increase over time in the lumbar DRGs indicating the development of nerve damage over time (Ivanavicius et al 2007, Orita et al 2011, Thakur et al 2012). Furthermore, activated microglia in the ipsilateral spinal cord has been reported following peripheral nerve damage, and so microgliosis may also indicate the development of neuropathy in this model (Calvo and Bennet 2012, Thakur et al 2012). This study will investigate the development of a neuropathic component in the 2mg MIA model further.

6.1.4 Gabapentin and pregabalin: effective analgesics for neuropathic pain

The gabapentinoid drugs, gabapentin and pregabalin, were originally designed as analogues of GABA, yet neither molecules demonstrated affinity for GABA receptors or produced significant effects on GABA levels (Jensen et al 2002). However, gabapentin and pregablin were found to bind to the $\alpha_2\delta$ subunits of VGCCs, with the highest affinity for the $\alpha_2\delta_1$ subunit, which are involved in the activation kinetics and trafficking of the channel to the plasma membrane (Davies et al 2007). Both gabapentin and pregablin have proved effective in animal models of neuropathy, and are now considered key therapies for relieving pain in neuropathic conditions (Patel and Dickenson 2016).

As discussed in Section 1.1.3.3, VGCCs are crucial for the transmission of the nociceptive signal from the periphery to the spinal cord, and translate the electrical nociceptive signal into a chemical one through triggering the release of neurotransmitters (Snutch 2005, Simms and Zamponi 2014). The $\alpha_2\delta_1$ subunit is responsible for enhancing the trafficking of VGCCs to the neuronal cell membrane, which subsequently increases the calcium current density, and as such the $\alpha_2\delta_1$ subunit can accelerate activation and inactivation of calcium currents (Dolphin 2013, Patel and Dickenson 2016). The $\alpha_2\delta_1$ subunit is expressed in the dorsal horn, predominantly presynaptically in superficial lamina, but there is also a moderate expression postsynaptically on neurons in the deeper lamina (Taylor and Garrido 2008). The $\alpha_2\delta_1$ subunit is also expressed in a subset of unmyleinated C fibre neurons within the DRGs (Taylor and Garrido 2008). Overall, the $\alpha_2\delta_1$ subunit regulates the trafficking of VGCCs to plasma membranes, thereby influencing calcium influx and the synaptic release of neurotransmitters (Patel and Dickenson 2016).

As discussed in Section 1.1.6, activity dependent central sensitisation can be mediated via activation of intracellular signaling cascades, which can subsequently activate transcriptional changes within neurons (Woolf and Salter 2000). One example of this is the upregulation of the $\alpha_2\delta_1$ subunit following neuropathy; increased levels of the $\alpha_2\delta_1$ subunit have been demonstrated in the dorsal horn and DRGs following nerve injury in rat SNL models (Figure 6.2) (Luo et al 2001, Newton et al 2001, Bauer et al 2009). Interestingly, uninjured transgenic mice overexpressing the $\alpha_2\delta_1$ subunit have reduced mechanical withdrawal thresholds, which are similar to those observed in SNL mice, yet this behavioural hypersensitivity was significantly attenuated by both N- and L-type calcium channel blockers (Chang et al 2015). Additionally, whole-cell patch clamping

recordings have demonstrated that spinal neurons overexpressing the $\alpha_2\delta_1$ subunit have an increased frequency of miniature excitatory post-synaptic currents (EPSC), which is mediated by AMPA and NMDA receptors (Nguyen et al 2009, Zhou and Lou 2014). Furthermore, $\alpha_2\delta_1^{-/-}$ knock-out mice show a delay in developing mechanical hypersensitivity following partial sciatic nerve ligation, yet a chronic pain state does eventually develop, suggesting that increased expression of the $\alpha_2\delta_1$ subunit is not crucial for the initiation and maintenance of central sensitisation (Patel et al 2013). These findings indicate that elevated levels of the $\alpha_2\delta_1$ subunit mediates an enhanced excitability of afferent fibres and increased release of pre-synaptic glutamate, which subsequently leads to reduced excitation thresholds of post-synaptic second order dorsal horn neurons (Nguyen et al 2009, Patel and Dickenson 2016). Overall, these mechanisms manifest as behavioural hypersensitivity.

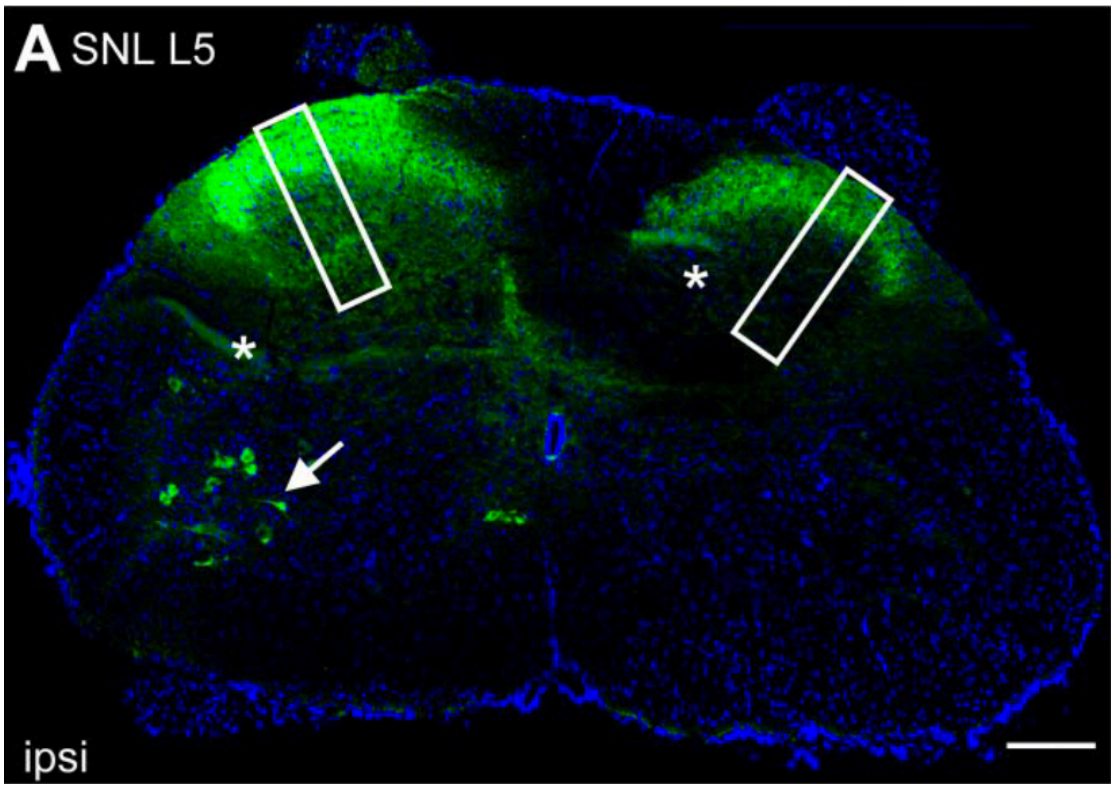


Figure 6.2. A cross-section of the L5 lumbar region of the spinal cord showing $\alpha_2\delta_1$ immunoreactivity from an SNL rat 24 days after surgery. The green staining represents $\alpha_2\delta_1$ subunit expression while blue represents nuclear counter-staining. There is a substantial increase in the level of staining for $\alpha_2\delta_1$ subunits in the dorsal horn ipsilateral to nerve injury. The arrow indicates an increase in $\alpha_2\delta_1$ subunit expression in ipsilateral motor neurons. (Taken from Bauer et al 2009).

Several studies indicate that $\alpha_2\delta_1$ subunit expression levels are increased following neuropathy and gabapentinoid drugs bind with high affinity to the $\alpha_2\delta_1$ subunit, supporting the concept that the $\alpha_2\delta_1$ subunit is the molecular target for mediating the

gabapentinoid drugs analgesic effects (Gee et al 1996, Fields et al 2006, Bauer et al 2009, Li et al 2011). The exact molecular mechanisms in which targeting the $\alpha_2\delta_1$ subunit inhibits the release of neurotransmitters are yet to be determined. The chronic application of Gabapentin inhibited voltage-gated Ca^{2+} influx in DRG neurons, yet acute application did not consistently reduce calcium currents (Sutton et al 2002, Hendrich et al 2008). Additionally, chronic systemic pregabalin has been demonstrated to inhibit the trafficking of the $\alpha_2\delta_1$ subunit to presynaptic terminals in the dorsal horn in SNL rats (Bauer et al 2009). This indicates that reduced trafficking of the $\alpha_2\delta_1$ subunit and the subsequent reduction in density of VGCCs, reduces calcium currents and therefore inhibits the release of neurotransmitters (Hendrich et al 2008). Given the time frame required for axonal trafficking effects, it is unlikely this mechanism is responsible for the analgesic effects of acute gabapentinoid treatment (Field et al 2006, Patel et al 2013). Therefore, there is likely distinct molecular mechanisms for the analgesic effects of acute and chronic gabapentinoid treatment.

Despite the $\alpha_2\delta_1$ subunit being the sole target for the analgesic actions of gabapentinoids, an upregulation of the $\alpha_2\delta_1$ subunit is not always crucial for gabapentinoid drugs to have effective analgesic actions (Suzuki et al 2005). The development of central sensitisation and the subsequent enhanced activity of descending brainstem facilitations acting on spinal 5-HT_3 receptors has been demonstrated to create the necessary conditions for gabapentinoid drugs to mediate analgesia (Suzuki et al 2005). Interestingly, when the spino-bulbo-spinal loop was disrupted in SNL rats, through ablation of spinal NK1 projection neurons or μ-opioid receptor expressing neurons in the RVM with the neurotoxin saporin, gabapentinoids could no longer inhibit the excitability of WDR neurons in the dorsal horn (Suzuki et al 2005, Bee and Dickenson 2008). Furthermore, the intrathecal administration of the 5-HT_3 receptor antagonist ondansetron attenuated tactile allodynia and thermal hyperalgesia in SNL mice and transgenic mice overexpressing the $\alpha_2\delta_1$ subunit (Chang et al 2013). Remarkably, when a 5-HT_3 receptor agonist was applied spinally in naïve rats, pregablin could inhibit spinal neurons even in the absence of injury (Suzuki et al 2005). These findings indicated that the activation of pre-synpatic 5-HT_3 receptors in the dorsal horn, due to central sensitisation and enhanced descending facilitations, creates adequate conditions for gabapentinoid drugs to inhibit calcium currents and subsequently prevent neurotransmitter release. This may imply interactions occur between $\alpha_2\delta_1$ subunits and descending brainstem controls acting at spinal 5-HT_3 receptors, and therefore gabapentinoids may be effective in non-neuropathic pain

conditions where there is no upregulation of the $\alpha_2\delta_1$ subunits (Patel and Dickenson 2016).

Overall, the gabapentinoid drugs are effective analgesic molecules in neuropathic conditions, which function by preventing the trafficking of $\alpha_2\delta_1$ subunits of VGCCs to the plasma membrane of active synapses in the dorsal horn, inhibiting voltage gated calcium currents and subsequently preventing the release of neurotransmitters. However, the gabapentinoids may also prove effective in non-neuropathic conditions when central sensitisation has developed and there is enhanced activation of spinal 5-HT_3 receptors due to an increased facilitatory drive from the brainstem.

6.1.4.1 The efficacy of gabapentin and pregabalin in patients

Both gabapentin and pregabalin have proved effective in patients with peripheral and central neuropathy (Wiffen et al 2005, Moore et al 2014a, Moore et al 2014b). In advanced OA cases, when articular cartilage is extensively damaged and destruction of the subchondral bone occurs, there may be the induction of neuropathic features (Thakur et al 2014). Therefore, gabapentinoid drugs may also prove effective in a subset of OA patients. Indeed, a combination therapy of pregabalin with the NSAID Meloxicam proved more effective at relieving pain in a cohort of patients with knee OA than pregabalin and Meloxicam treatments alone (Ohtori et al 2013). The authors concluded that both inflammatory and neuropathic elements contributed to the pain associated with knee OA in these patients (Ohtori et al 2013). This indicates further that if neuropathic components are identified in patients with OA, they are likely to benefit from gabapentinoid analgesics.

6.1.4.2 The efficacy of gabapentin and pregabalin in animal models

Both gabapentin and pregabalin have been shown to have analgesic actions in the MIA model of OA (Ivanavicius et al 2007, Rahman et al 2009, Vonsy et al 2009, Thakur et al 2012). Chronic gabapentin treatment has been demonstrated to attenuate cooling hypersensitivity, reduce ambulatory-evoked pain scores, and weight-bearing asymmetry in rats 14 days after MIA injection (Ivanavicius et al 2007, Vonsy et al 2009). Electrophysiological recordings of WDR neurons in the 2mg MIA rat model have demonstrated that systemic pregabalin significantly reduced mechanically and thermally evoked neuronal responses, while acute pregabalin treatment did not have this effect in saline injected sham controls (Rahman et al 2009, Thakur et al 2012). In addition, despite an elevated expression of $\alpha_2\delta_1$ mRNA in L3-L6 DRGs from MIA animals,

the inhibitory actions of pregabalin on spinal neurons was blocked by spinal pre-administration of the 5-HT_3 receptor antagonist ondansetron. This indicates that even with elevated levels of $\alpha_2\delta_1$, an enhanced descending faciliatory drive acting at spinal 5-HT_3 receptors is required for pregabalins analgesic actions (Rahman et al 2009). As pregabalin has proved effective in the MIA model and involves an enhanced serotonergic drive acting at 5-HT_3 receptors in the spinal cord, which is linked to the loss of DNIC in chronic pain states, this study will investigate the efficacy of pregabalin and its subsequent effect on DNIC expression in the 2mg MIA model (Bannister et al 2015).

6.1.5 Chapter aims

This study aimed to further investigate the descending pathways involved in the expression of DNIC. Firstly, through pharmacologically manipulating noradrenaline levels and blocking the α_2-adrenoceptors. Secondly, through pharmacologically modulating the activity of the 5-HT_7 receptor with a selective antagonist and agonist in the early and late phase MIA animals respectively. Further, any pharmacological effects due to upregulation of the noradrenergic and serotonergic receptors in the spinal cord or DRGs was explored. A neuropathic component to the 2mg MIA model was investigated through assessing the innervation of the knee with a neuronal tracer and analyzing expression of the neuronal damage marker ATF-3 in lumbar DRGs. The efficacy of Pregabalin was assessed and compared in the early and late phase MIA model, and its effect on the expression of DNIC was investigated. Finally, the inhibitory effect on mechanically evoked spinal neuronal firing in response to of a combination therapy of Tapentadol and Pregabalin was assessed. Overall, this study aimed to pharmacologically restore DNIC in late phase MIA animals to better understand the alterations in descending controls that may mediate its loss, and to better understand the neuropathic component of the 2mg MIA model.

6.2 Methods

6.2.1 Electrophysiology

Electrophysiological recordings were taken as previously described (See Section 2.1); in early phase animals this was 2-6 days post MIA injection, and for late phase animals this was 14-20 days post MIA injection. For pharmacology experiments, extracellular recordings were taken from WDR neurons in the ipsilateral dorsal horn relative to MIA injection.

6.2.2 Pharmacology

Early phase MIA animals

- **SB-269970** (Tocris) is a 5-HT_7 receptor antagonist. This was dissolved in saline and applied topically (100µg/50µL) to the spinal cord.

- **Atipamezole** (Sigma) is an α_2-adrenoceptor antagonist. Atipamezole was dissolved in vehicle (97% normal saline, 2% cremaphor, 1% DMSO) and a concentration of (100µg/50µL) was applied topically to the spinal cord.

- **Pregabalin** (gifted from Pfizer) is an analogue of Gabapentin. Pregabalin was dissolved in normal saline to a concentration of 10mg/ml. This is then injected subcutaneously with the volume dependent on the weight of the animal such that the dose delivered was always 10mg/kg.

Early phase Sham control animals received an intra-articular injection of 25µL saline, with electrophysiology and pharmacology carried out 2-6 days post injection.

Late Phase

- **AS-19** (Tocris) is a selective 5-HT_7 receptor agonist. This was dissolved in vehicle (97% normal saline, 2% cremaphor, 1% DMSO) and applied topically to the spinal cord (100µg/50µL).

- **Tapentadol** (gifted from Grunenthal) has a dual mode of action; it is a μ opiod receptor agonist and a noradrenaline reuptake inhibitor. This was dissolved in normal saline and injected subcutaneously at 3 doses; 1mg/kg, 2mg/kg and 5mg/kg.

- **Pregabalin** (Pfizer) was dissolved in normal saline and injected subcutaneously to give a systemic dose of 10mg/kg.

Late phase Sham control animals received an intra-articular injection of 25μL saline, with electrophysiology and pharmacology carried out 14-20 days post injection.

6.2.3 Immunohistochemistry

The lumbar spinal cord and the L3-L5 DRGs ipsilateral to MIA injection were taken from early and late phase MIA and sham animals. For early phase animals tissues were removed on day 4 post-injection, for late phase animals tissues were removed on day 14 post-injection. The tissue was processed as described in Section 2.6.

6.2.4 qPCR

The ipsilateral lumbar dorsal horn and ipsilateral L3-L5 DRGs were taken from MIA and sham animals. For early phase MIA and sham animals the tissue was taken 4 days post-injection. For late phase MIA and sham animals the tissue was taken 14 days post-injection. The tissue was processed as discussed in Section 2.7.

6.2.5 Statistical analysis

For a description of the statistical tests used for analysis please refer to section 2.8.

6.3 Results

6.3.1 The role of the 5-HT$_7$ receptor in the expression of DNIC in the MIA model

The loss of DNIC observed in late phase MIA animals may develop due to an imbalance in descending inhibitory and facilitatory descending controls arising in the brainstem, which is similar to that observed in a SNL rat model of neuropathy (Bannister et al 2015). As discussed in Chapter 4, increased levels of serotonin acting upon the 5-HT$_7$ receptor in the spinal cord is sufficient to restore DNIC in a SNL rat model of neuropathy (Bannister et al 2017). Therefore, the role played by the 5-HT$_7$ receptor in the expression of DNIC was assessed further in early and late phase MIA animals.

Firstly, as a functioning DNIC system was present in early phase MIA animals, the actions of serotonin acting at the 5-HT$_7$ receptor were blocked using the selective 5-HT$_7$ receptor antagonist SB-2669970. Following spinal administration of the 5-HT$_7$ receptor antagonist, the resultant DNIC expression was assessed in order to investigate the role played by the 5-HT$_7$ receptor when the DNIC system is functional.

In early phase MIA animals, the 5-HT$_7$ receptor antagonist reduced the DNIC expression with a concurrent noxious ear and knee pinch but did not abolish it. Following spinal application of the 5-HT$_7$ receptor antagonist, the inhibition of mechanically evoked neuronal firing induced by DNIC following application of a concurrent noxious ear pinch fell from approximately 30% for all mechanical stimulations to 15%, 21% and 19% for 8g, 26g and 60g stimulations respectively in early phase MIA animals (Figure 6.3A). Furthermore, the neuronal inhibition induced by DNIC with a concurrent noxious knee pinch fell to 35%, 16%, and 17% with 8g, 26g, and 60g stimulations respectively in early phase MIA animals (Figure 6.3A). Therefore, the level of neuronal inhibition was reduced by approximately 10% when the actions of serotonin acting at 5-HT$_7$ receptors in the spinal cord was blocked, yet the reduction in neuronal firing induced by a concurrent noxious ear or knee pinch was still significant compared to pre-conditioned responses (Figure 6.3A). Similarly, the level of mechanically evoked neuronal inhibition was reduced but not abolished in early phase saline injected sham control animals (Figure 6.3C). Overall, the spinal application of the 5-HT$_7$ receptor antagonist had no significant effect on pre-conditioned baseline mechanically evoked neuronal responses in either early phase MIA or sham animals (Figure 6.3B and 6.3D). As the 5-HT$_7$ receptor

reduced but did not block the expression of DNIC in both early phase MIA and sham animals, it indicates that while 5-HT_7 receptors in the spinal cord may play an inhibitory role involved with the induction of DNIC, other receptors in the spinal cord must also be responsible for full DNIC expression.

Secondly, as DNIC is abolished in late phase MIA animals, it was investigated if activation of spinal 5-HT_7 receptors with the selective agonist AS-19 was sufficient to restore DNIC. The actions of the spinal application of the selective 5-HT_7 receptor agonist AS-19 on the expression of DNIC was also assessed in late phase saline injected sham controls where the DNIC system was fully functional.

In late phase MIA injected animals, the spinal application of the 5-HT_7 agonist restored DNIC such that a significant reduction in mechanically evoked neuronal firing was achieved with concurrent noxious ear and knee pinch (Figure 6.3E). Following the spinal application of the 5-HT_7 receptor agonist in late phase MIA animals, the mechanically evoked neuronal responses to 8g, 26g, and 60g stimulations was reduced by 28%, 34%, and 32% with a concurrent noxious ear pinch, and 51%, 31%, and 25% with a concurrent noxious knee pinch (Figure 6.3E). Furthermore, the spinal application of the 5-HT_7 receptor agonist in late phase MIA animals significantly reduced pre-conditioned mechanically evoked neuronal firing.

In late phase saline injected sham controls, the pre-drug level of neuronal inhibitions were 48%, 44%, and 40% induced with a concurrent noxious ear pinch, and 54%, 42%, and 46% induced with a concurrent noxious knee pinch in response to 8g, 26g and 60g mechanical stimulations respectively (Figure 6.3G). Following spinal application of the 5-HT_7 receptor agonist, the levels of neuronal inhibition were 37%, 45%, and 37% induced with a concurrent noxious ear pinch, and 58%, 54%, and 42% induced with a concurrent noxious knee pinch in response to 8g, 26g and 60g mechanical stimulations respectively (Figure 6.3G). This indicates that the levels of neuronal inhibition induced by DNIC did not appear to alter consistently following the spinal activation of 5-HT_7 receptors. Additionally, the spinal application of the 5-HT_7 receptor agonist did not significantly affect the pre-conditioned mechanically evoked neuronal firing (Figure 6.3F). Overall, these findings indicate that when DNIC is functional, as is the case in saline injected sham controls, further activation of spinal 5-HT_7 receptors does not result in an increased level of inhibition.

As the spinal application of the 5-HT$_7$ receptor agonist restores DNIC in late phase MIA animals but has limited effects in late phase saline injected sham controls, it indicates the function of the 5-HT$_7$ receptor may become altered in states of chronic pain. Furthermore, alterations in the activation properties of spinal 5-HT$_7$ receptors may be involved in the development of an abolished DNIC system in the late phase MIA animals.

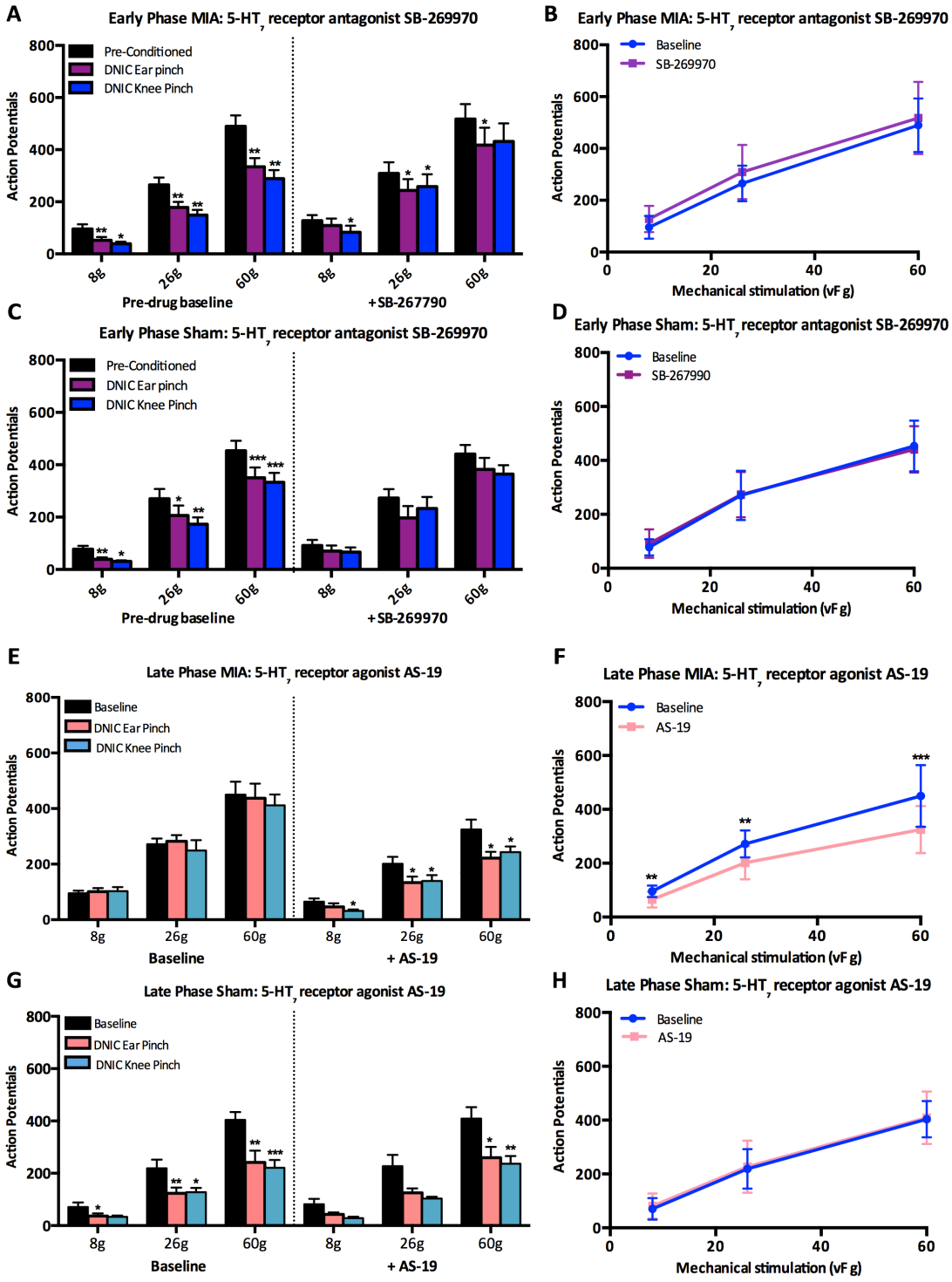


Figure 6.3. The involvement of the 5-HT$_7$ receptor in the expression of DNIC in early and late phase MIA and sham animals. A) The spinal application of the 5-HT$_7$ receptor antagonist SB-269970 in early phase MIA animals reduced the level of DNIC induced by concurrent noxious

ear and knee pinch but did not abolish it (n=6). B) The spinal application of the 5-HT_7 receptor antagonist SB-269970 in early phase MIA animals did not affect the pre-conditioned mechanically evoked neuronal responses. C) The spinal application of the 5-HT_7 receptor antagonist SB-269970 in early phase sham animals reduced the level of neuronal inhibition induced by DNIC but did not abolish it (n=6). D) The spinal application of the 5-HT_7 receptor antagonist SB-269970 in early phase sham animals did not affect the pre-conditioned mechanically evoked neuronal responses. E) The spinal application of the 5-HT_7 receptor agonist AS-19 in late phase MIA animals restored neuronal inhibition induced by a concurrent noxious ear or knee pinch (n=6). F) The spinal application of the 5-HT_7 receptor agonist AS-19 in late phase MIA animals significantly reduced pre-conditioned mechanically evoked neuronal responses. G) The spinal application of the 5-HT_7 receptor agonist AS-19 in late phase sham animals had little effect on the level of mechanically evoked neuronal inhibition induced by a concurrent noxious ear or knee pinch (n=5). H) The spinal application of the 5-HT_7 receptor agonist AS-19 in late phase sham animals did not effect the pre-conditioned mechanically evoked neuronal responses. Two-way ANOVA with Bonferroni correction. (*P<0.05, **P<0.01, ***P<0.001).

6.3.2 The involvement of the descending noradrenergic system in the expression of DNIC in the MIA model

It has been demonstrated in Chapter 4 and in previous studies that the full expression of DNIC is reliant upon an inhibitory descending noradrenergic system acting at α_2-adrenergic receptors in the spinal cord (Bannister et al 2015, Bannister et al 2017). Therefore, the requirement of noradrenaline acting at α_2-adrenergic receptors in the spinal cord for the expression of DNIC was assessed in early phase MIA and sham animals, where the DNIC system was functional. Specifically, the actions of noradrenaline at α_2-adrenergic receptors in the spinal cord were blocked with the α_2-adrenergic receptor antagonist atipamezole and the resultant DNIC expression was assessed in early phase MIA and sham animals.

In early phase MIA animals, the spinally applied α_2-adrenergic receptor antagonist blocked the DNIC effect, such that there was no significant reduction of mechanically evoked neuronal firing with either a concurrent noxious ear or knee pinch (Figure 6.4A). The spinal application of the α_2-adrenergic receptor antagonist also resulted in facilitated pre-conditioned responses, with a significantly increased number of action potentials fired in response to 8g, 26g and 60g (Figure 6.4B). Similarly, the spinal application of the α_2-adrenergic receptor antagonist blocked the DNIC effect in early phase sham animals. There was no significant inhibition of mechanically evoked neuronal firing with a concurrent noxious ear pinch, and no significant reduction of 26g and 60g mechanically evoked neuronal firing with a concurrent noxious knee pinch (Figure 6.4C). However, there was still a significant 23% reduction in neuronal firing with a concurrent noxious knee pinch in response to the 8g stimulation (Figure 6.4C). Interestingly, the spinal application of the α_2-adrenergic receptor antagonist had no

significant effect on the pre-conditioned mechanically evoked neuronal responses in early phase saline injected sham controls (Figure 6.4D). As the spinal application of the α_2-adrenergic receptor antagonist facilitates neuronal responses in early phase MIA animals but not in saline injected controls, it may indicate an enhanced inhibitory noradrenergic descending system in the early MIA state.

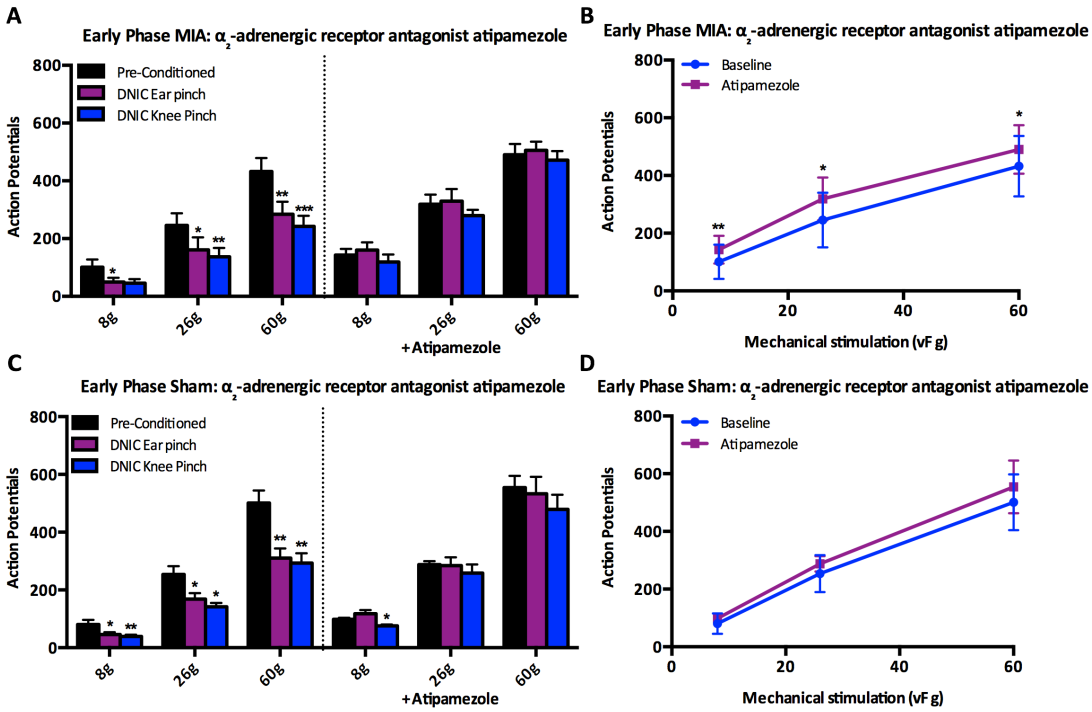


Figure 6.4. The role played by the inhibitory noradrenergic descending system in the expression of DNIC in the early phase MIA model. A) The spinal application of the α_2-adrenergic receptor antagonist atipamezole blocks the inhibitory effect on mechanically evoked neuronal firing induced by DNIC in early phase MIA animals (n=5). B) The spinal application of the α_2-adrenergic receptor antagonist atipamezole facilitates the pre-conditioned mechanically evoked neuronal responses in early phase MIA animals (n=5). C) The spinal application of the α_2-adrenergic receptor antagonist atipamezole blocks the inhibitory effect on neuronal firing induced by a concurrent noxious ear pinch to all mechanical stimulations and by a concurrent noxious knee pinch in response to 26g and 60g mechanical stimulations in early phase sham animals (n=5). The spinal application of the α_2-adrenergic receptor antagonist atipamezole has no effect on the pre-conditioned mechanically evoked neuronal responses in early phase saline injected sham controls (n=5). Two-way ANOVA with Bonferroni correction. (*P<0.05, **P<0.01, ***P<0.001).

6.3.2.1 The actions of Tapentadol in late phase MIA animals

Tapentadol has a dual mode of action, acting as both a NRI and an agonist for the MOR. As the DNIC system has been reported to rely upon descending noradrenergic inhibitory controls, but also have a partly opioid component, the actions of Tapentadol were assessed in late phase MIA animals and saline injected sham controls (Le Bars et al 1981, Bannister et al 2015, Bannister et al 2017). Specifically, it was investigated if the

combination of increased levels of synaptic noradrenaline and MOR agonism were sufficient to restore DNIC in late phase MIA animals.

All of the doses of Tapentadol tested (1mg/kg, 2mg/kg and 5mg/kg) restored the inhibitory effect on mechanically evoked neuronal inhibition induced by either a concurrent noxious ear or knee pinch (Figure 6.5). Following a systemic dose of 1mg/kg Tapentadol, the level of neuronal inhibition induced by a concurrent noxious ear pinch was 6%, 22% and 27% and by a concurrent noxious knee pinch was 31%, 22% and 28% with 8g, 26g and 60g mechanical stimulations respectively (Figure 6.5A). Following a systemic dose of 2mg/kg Tapentadol, the level of neuronal inhibition induced by a concurrent noxious ear pinch was 21%, 31% and 31% and by a concurrent noxious knee pinch was 10%, 28% and 32% with 8g, 26g and 60g mechanical stimulations respectively (Figure 6.5C). Following a systemic dose of 5mg/kg Tapentadol, the level of neuronal inhibition induced by a concurrent noxious ear pinch was 25%, 33%, and 31% and by a concurrent noxious knee pinch was 16%, 33% and 32% with 8g, 26g and 60g mechanical stimulations respectively (Figure 6.5E). Interestingly, for 1mg/kg of Tapentadol there was only a significant reduction of neuronal firing induced by DNIC in response to the most noxious 60g mechanical stimulation, while for 2mg/kg and 5mg/kg Tapentadol there was a significant reduction of neuronal firing induced by DNIC in response to 26g and 60g mechanical stimulations (Figure 6.5A, 6.5C, and 6.5E). At no Tapentadol dose was the inhibitory effect on 8g mechanically evoked neuronal firing significant. Additionally, Tapentadol dose-dependently reduced pre-conditioned mechanically evoked neuronal firing (Figure 6.5H). This indicates that systemic Tapentadol is sufficient, even at low doses, to restore the endogenous inhibitory system required for DNIC expression.

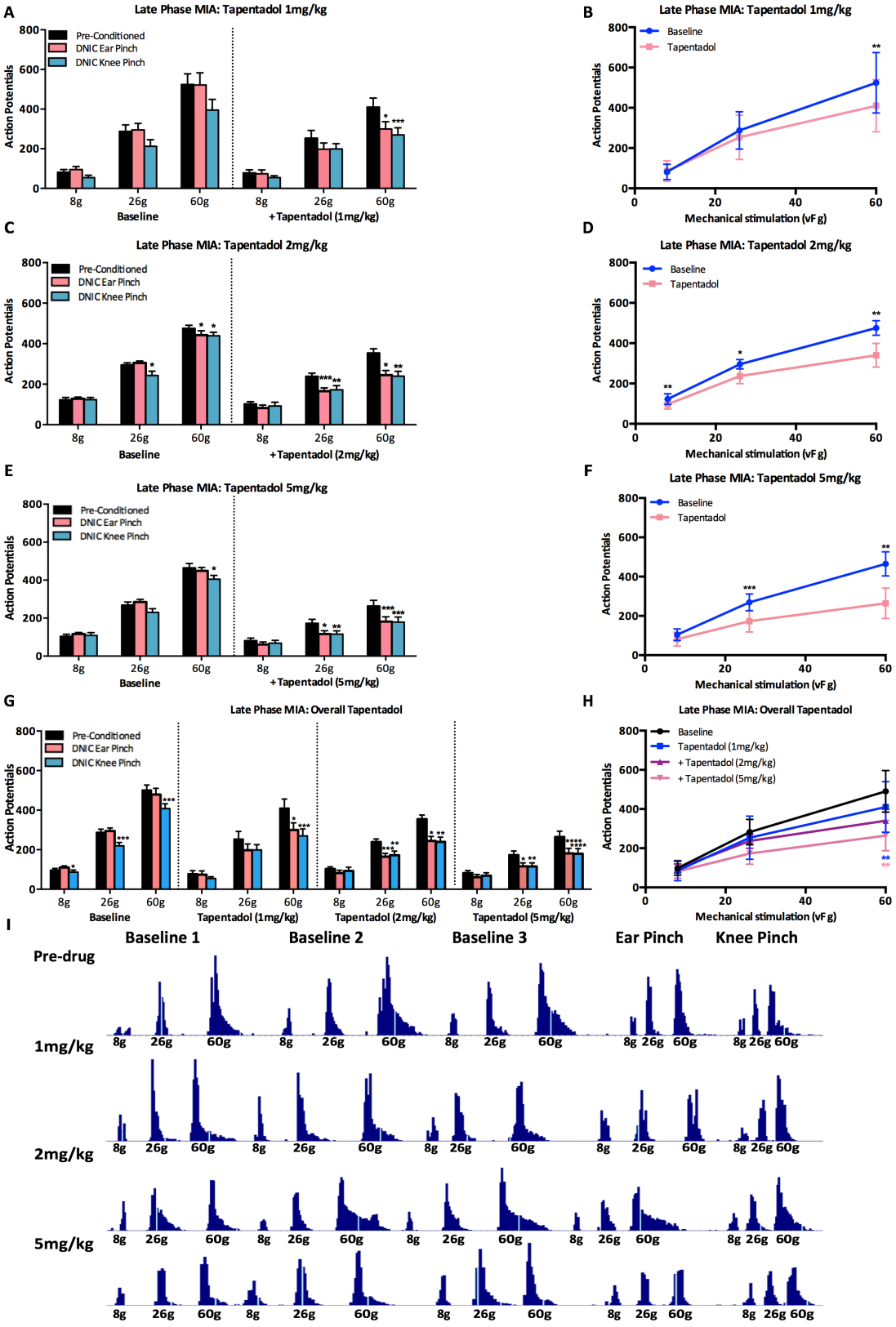


Figure 6.5 The dose-dependent effects of tapentadol on mechanically evoked neuronal firing and DNIC expression. A) A 1mg/kg tapentadol dose restores the inhibitory effect of DNIC induced by a concurrent noxious ear or knee pinch, such that there is a significant reduction of mechanically evoked neuronal firing with a 60g stimulation (n=8). B) A 1mg/kg systemic tapentadol dose significantly inhibits pre-conditioned mechanically evoked neuronal responses to a 60g stimulation. C) A 2mg/kg tapentadol dose restores the inhibitory effect of DNIC induced

by a concurrent noxious ear or knee pinch, such that there is a significant reduction in mechanically evoked neuronal firing in response to 26g and 60g stimulations (n=6). D) A 2mg/kg systemic tapentadol dose significantly inhibits pre-conditioned mechanically evoked neuronal responses. E) A 5mg/kg systemic tapentadol dose restores the inhibitory effect of DNIC induced by a concurrent noxious ear or knee pinch, such that there is a significant reduction of mechanically evoked neuronal firing in response to 26g and 60g stimulations (n=7). F) A 5mg/kg systemic tapentadol dose significantly inhibits pre-conditioned mechanically evoked neuronal responses to 26g and 60g stimulations. G) Increasing doses of systemic tapentadol increasingly inhibit pre-conditioned mechanically evoked neuronal responses and increase the level of neuronal inhibition induced with DNIC. H) tapentadol dose-dependently inhibits pre-conditioned mechanically evoked neuronal responses. I) A raw trace from one example WDR neuron, indicating the pre-drug baseline DNIC trial consisting of 3 baseline pre-conditioned responses, one DNIC response with a concurrent noxious ear pinch and one DNIC response with a concurrent noxious knee pinch. This trace also indicates a DNIC trial following a 1mg/kg systemic tapentadol dose, a DNIC trial following a 2mg/kg systemic tapentadol dose, and a DNIC trial following a 5mg/kg tapentadol dose. Two-way ANOVA with Bonferroni correction. *P<0.05, **P<0.01, ***P<0.001.

The effects of Tapentadol on mechanically evoked neuronal firing rates and DNIC expression were also explored in saline injected sham control animals. While all doses of systemic Tapentadol inhibited pre-conditioned mechanically evoked neuronal firing (Figure 6.6H), it had no consistent effect on the level of neuronal inhibition induced by DNIC. Following a systemic dose of 1mg/kg Tapentadol, the level of neuronal inhibition induced by a concurrent noxious ear pinch changed from pre-drug levels of 48%, 31%, and 34% to 46%, 29%, and 31% and by a concurrent noxious knee pinch from 49%. 38%, and 46% to 36%, 34% and 29% in response to 8g, 26g and 60g mechanical stimulations respectively (Figure 6.6A). The 2mg/kg dose of Tapentadol resulted in the level of neuronal inhibition induced by a concurrent noxious ear pinch changing from pre-drug levels of 45%, 44%, and 40% to 35%, 40%, and 29% and with a concurrent noxious knee pinch from 50%, 42%, and 43% to 50%, 36% and 28% in response to 8g, 26g, and 60g mechanical stimulations respectively (Figure 6.6C). The 5mg/kg dose of Tapentadol resulted in the level of neuronal inhibition induced by a concurrent noxious ear pinch changing from pre-drug levels of 47%, 35%, and 28% to 45%, 38%, and 32% and with a concurrent noxious knee pinch from 57%, 36% and 37% to 43%, 25% and 28% in response to 8g, 26g and 60g mechanical stimulations respectively (Figure 6.6E). This indicates that when the endogenous inhibitory system serving DNIC is functional, as is the case in late phase saline injected sham controls, increased synaptic levels of noradrenaline and MOR agonism have a limited effect on the level of inhibition induced by DNIC.

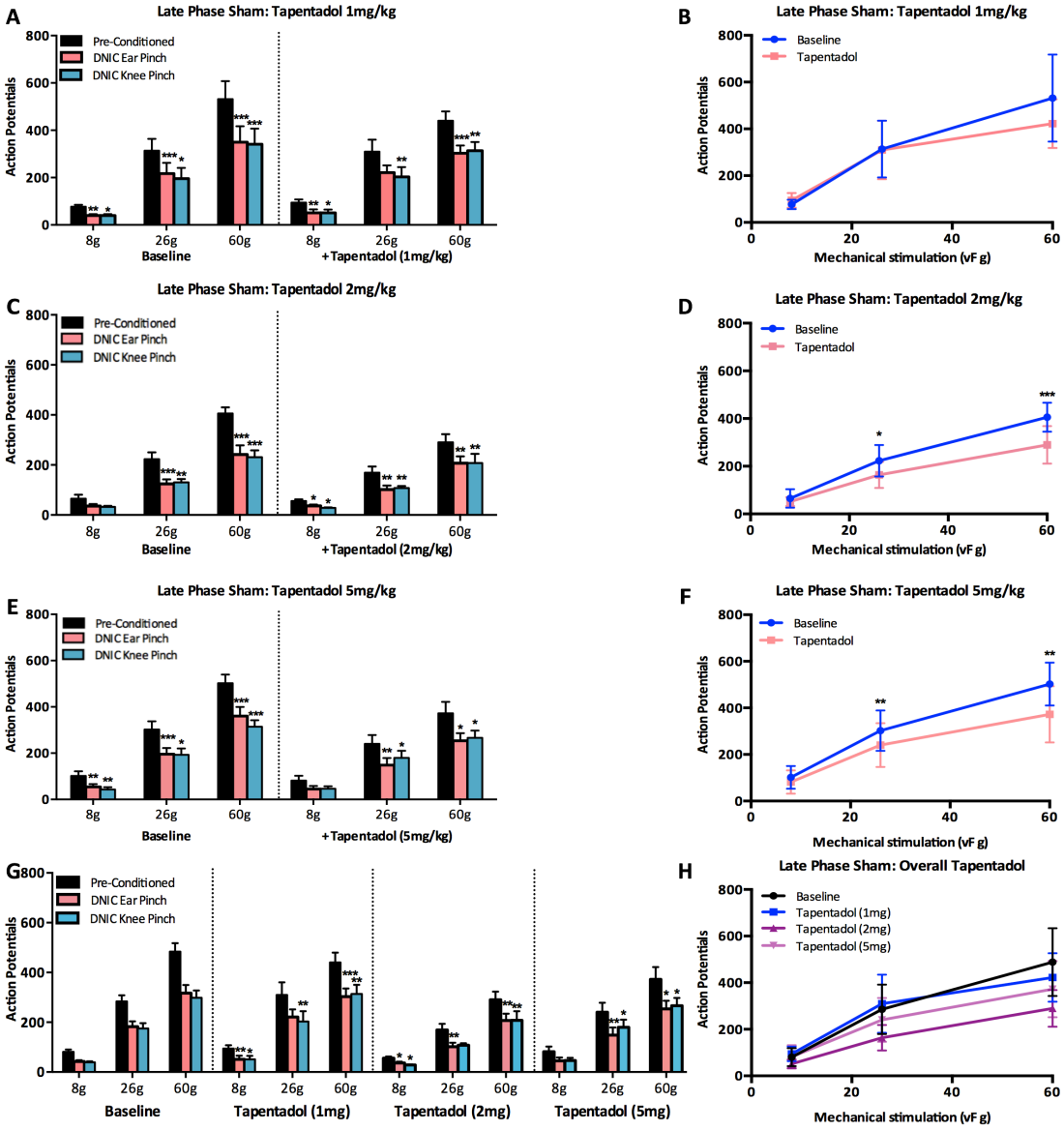


Figure 6.6 The effects of tapentadol on mechanically evoked neuronal firing and DNIC expression in late phase saline injected sham controls. A) There was a significant reduction in mechanically evoked neuronal firing induced by DNIC with a concurrent noxious ear or knee pinch both before and after a systemic dose of 1mg/kg tapentadol (n=6). B) A 1mg/kg systemic dose of tapentadol does not significantly reduce pre-conditioned mechanically evoked neuronal firing. C) There was a significant reduction in mechanically evoked neuronal firing induced by DNIC with a concurrent noxious ear or knee pinch in response to 26g and 60g stimulations both before and after a systemic dose of 2mg/kg tapentadol (n=6). D) A 2mg/kg systemic dose of tapentadol significantly inhibited 26g and 60g mechanically evoked neuronal firing. E) There was a significant reduction in mechanically evoked neuronal firing induced by DNIC with a concurrent noxious ear or knee pinch before drug was injected, following a 5mg/kg systemic dose of tapentadol there was a significant reduction in mechanically evoked neuronal firing induced by DNIC with a concurrent noxious ear or knee pinch in response to 26g and 60g stimulations (n=6). F) A 2mg/kg systemic dose of tapentadol significantly inhibited mechanically evoked neuronal firing. G) Increasing doses of systemic tapentadol has limited effect on the level of neuronal inhibition induced by DNIC in saline injected sham controls. H) All doses of tapentadol (1mg/kg, 2mg/kg, and 5mg/kg) reduce mechanically evoked neuronal firing but this does not reach significance. Two-way ANOVA with Bonferroni correction. *P<0.05, **P<0.01, ***P<0.001.

6.3.3 The expression of noradrenergic and serotonergic receptors in the spinal cord

As discussed in detail in Chapter 4, the late phase MIA animals having an abolished DNIC system indicates an imbalance in descending inhibitory and facilitatory controls, specifically a reduction of descending noradrenergic controls acting at α_2-adrenergic receptors and enhanced descending serotonergic controls acting at 5-HT_3 receptors in the spinal cord. One mechanism through which this imbalance may be mediated could be an increase or decrease in the expression of receptors in the dorsal horn, such that the release of monoaminergic neurotransmitters into the spinal cord will subsequently produce larger or smaller effects. In addition, an increase in 5-HT_7 receptor density has been demonstrated in the ipsilateral dorsal horn of mice with partial sciatic nerve ligation, suggesting a compensatory mechanism whereby receptor expression is increased to mediate inhibition (Brenchat et al 2010). Therefore, the mRNA expressions of the α_2-adrenergic, 5-HT_3, and 5-HT_7 receptors in the lumbar dorsal horn and DRGs were investigated.

There was no significant difference in the mRNA expression of α_2-adrenergic, 5-HT_3, and 5-HT_7 receptors in the ipsilateral lumbar dorsal horn of the spinal cord between early phase and late phase MIA animals and their respective saline-injected sham control groups (Figure 6.7A). Additionally, there was no significant difference in the mRNA expression of α_2-adrenergic, 5-HT_3, and 5-HT_7 receptors in the lumbar DRGs L3-L5 between early phase and late phase MIA animals and their respective saline-injected sham control groups (Figure 6.7H). The lack of changes in the mRNA expression of these noradrenergic and serotonergic receptor subtypes indicates that the imbalance in inhibitory and faciliatorty descending controls is not a result of an upregulated or downregulated expression of receptors in the spinal cord. Therefore, the reduced inhibitory noradrenergic descending controls and enhanced facilitatory serotonergic descending controls likely occur through changes in volume transmission (Todd 2010).

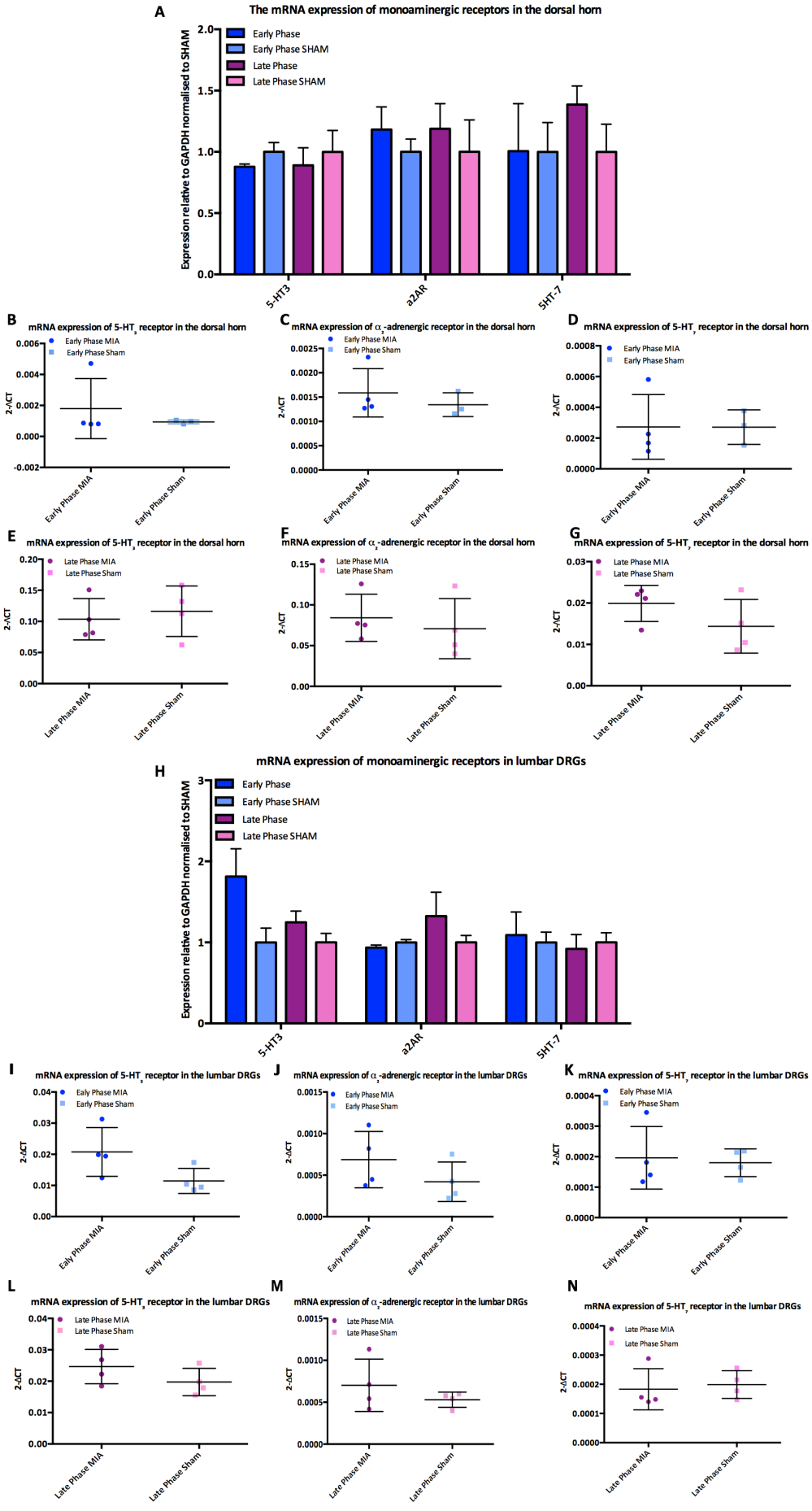

A
The mRNA expression of monoaminergic receptors in the dorsal horn
Expression relative to GAPDH normalised to SHAM
Early Phase
Early Phase SHAM
Late Phase
Late Phase SHAM
5-HT3
a2AR
5HT-7
B
mRNA expression of 5-HT3 receptor in the dorsal horn
C
mRNA expression of α2-adrenergic receptor in the dorsal horn
D
mRNA expression of 5-HT7 receptor in the dorsal horn
Early Phase MIA
Early Phase Sham
2-ΔCT
E
mRNA expression of 5-HT3 receptor in the dorsal horn
F
mRNA expression of α2-adrenergic receptor in the dorsal horn
G
mRNA expression of 5-HT7 receptor in the dorsal horn
Late Phase MIA
Late Phase Sham
H
mRNA expression of monoaminergic receptors in lumbar DRGs
I
mRNA expression of 5-HT3 receptor in the lumbar DRGs
J
mRNA expression of α2-adrenergic receptor in the lumbar DRGs
K
mRNA expression of 5-HT7 receptor in the lumbar DRGs
Ealy Phase MIA
Ealy Phase Sham
2-ΔCT
L
mRNA expression of 5-HT3 receptor in the lumbar DRGs
M
mRNA expression of α2-adrenergic receptor in the lumbar DRGs
N
mRNA expression of 5-HT7 receptor in the lumbar DRGs

Figure 6.7 The mRNA expression of monoaminergic receptors in the ipsilateral lumbar dorsal horn and DRGs. A) The mRNA expression of the 5-HT_3, α_2-adrenergic, and 5-HT_7 receptors in the ipsilateral lumbar dorsal horn relative to the housekeeping gene GAPDH and normalized to the relative saline injected sham control group (EP n=4, EPS n=3, LP n=4, LPS n=4). B) The mRNA expression of the 5-HT_3 receptor in the ipsilateral lumbar dorsal horn relative to GAPDH in early phase MIA animals. C) The mRNA expression of the α_2-adrenergic receptor in the ipsilateral lumbar dorsal horn relative to GAPDH in early phase animals. D) The mRNA expression of the 5-HT_7 receptor in the ipsilateral lumbar dorsal horn relative to GAPDH in early phase animals. E) The mRNA expression of the 5-HT_3 receptor in the ipsilateral lumbar dorsal horn relative to GAPDH in late phase animals. F) The mRNA expression of the α_2-adrenergic receptor in the ipsilateral lumbar dorsal horn relative to GAPDH in late phase animals. G) The mRNA expression of the 5-HT_7 receptor in the ipsilateral lumbar dorsal horn relative to GAPDH in late phase animals. H) The mRNA expression of the 5-HT_3, α_2-adrenergic, and 5-HT_7 receptors in the ipsilateral lumbar L3-L5 DRGs relative to the housekeeping gene GAPDH and normalized to the relative saline injected sham control group (EP n=4, EPS n=4, LP n=4, LPS n=4). I) The mRNA expression of the 5-HT_3 receptor in the ipsilateral L3-L5 DRGs relative to GAPDH in early phase animals. J) The mRNA expression of the α_2-adrenergic receptor in the ipsilateral L3-L5 DRGs relative to GAPDH in early phase animals. K) The mRNA expression of the 5-HT_7 receptor in the ipsilateral L3-L5 DRGs relative to GAPDH in early phase animals. L) The mRNA expression of the 5-HT_3 receptor in the ipsilateral L3-L5 DRGs relative to GAPDH in late phase animals. M) The mRNA expression of the α_2-adrenergic receptor in the ipsilateral L3-L5 DRGs relative to GAPDH in late phase animals. N) The mRNA expression of the 5-HT_7 receptor in the ipsilateral L3-L5 DRGs relative to GAPDH in late phase animals. For mRNA expressions relative to the saline injected sham controls groups (A and H), statistical significance was tested with a Kruskall-Wallis independent samples one-way ANOVA was used. For mRNA expression expressed as ΔCT values a significant difference was tested between the MIA group and saline injected sham controls with an independent T-test.

6.3.4 The innervation of the knee

As previously discussed (Section 1.3.4), while the cartilage is not innervated all other subchondral joint structures, including the synovium and subchondral bone are (Schaible et al 2009). Therefore any damage to these joint structures has the potential to cause a component of peripheral neuropathy. To investigate the innervation of the knee further, the neuronal tracer FastBlue was injected into the intrarticular space and the number and size of peripheral afferent fibres taking up the neuronal tracer were examined. Furthermore, this experiment assessed in which DRGs these peripheral afferents were terminating, to improve the understanding of where the joint damage induced barrage of nociceptive information may be projecting to. Finally, the ventral horn was examined to assess if any motor neurons surrounding the knee had taken up the neuronal tracer as a result of injection leakage.

Peripheral afferent fibres that took up the Fast Blue projected from L2 to S1 DRGs, with the highest number of peripheral afferents terminating in the L4 DRG (Figure 6.8A). While the L2 and S1 DRGs did show some positive Fast Blue staining, only 0.24% of neurons in the L2 DRGs and 0.28% of neurons in the S1 DRG showed positive Fast Blue

staining (Figure 6.8B). The L4 and L5 DRGs had a significantly higher percentage of neurons showing positive staining for Fast Blue, with 5.7% of neurons in the L4 DRG taking up Fast Blue and 3.4% of neurons in the L5 DRG taking up Fast Blue (Figure 6.8B). Overall, this indicates that peripheral afferent fibres innervating the knee project mainly to the L3, L4, L5 and L6 DRGs.

As the L3, L4, L5 and L6 DRGs had the highest percentage of neurons taking up the Fast Blue neuronal tracer, the size distribution of the total neurons per DRG were compared to the size distribution of those taking up Fast Blue to identify what type of peripheral fibres were innervating the knee. Cumulative sum analysis indicated that for L3, L4, L5 and L6 DRGs the size distribution for Fast Blue positive neurons was very similar to the size distribution of the total neurons within the DRG (Figure 6.8C, 6.8D, 6.8E and 6.8F). This indicates that both small and large peripheral afferent fibres were taking up the Fast Blue, this suggests that both A and C fibres innervate the knee.

Furthermore, Fast Blue staining was investigated in the lumbar ventral horn to investigate if any motor neurons surrounding the knee were taking up the neuronal tracer as this may indicate leakage of the injection to outside the intrarticular space. There no were no motor neurons showing positive Fast Blue staining in any of the ventral horn slices investigated (Figure 6.9). The lack of Fast Blue staining observed in motor neurons indicates that the injection was unlikely to be leaking from the intrarticular space. Therefore any positive Fast Blue staining observed in the DRGs was due to peripheral afferents directly innervating the knee taking up the neuronal tracer.

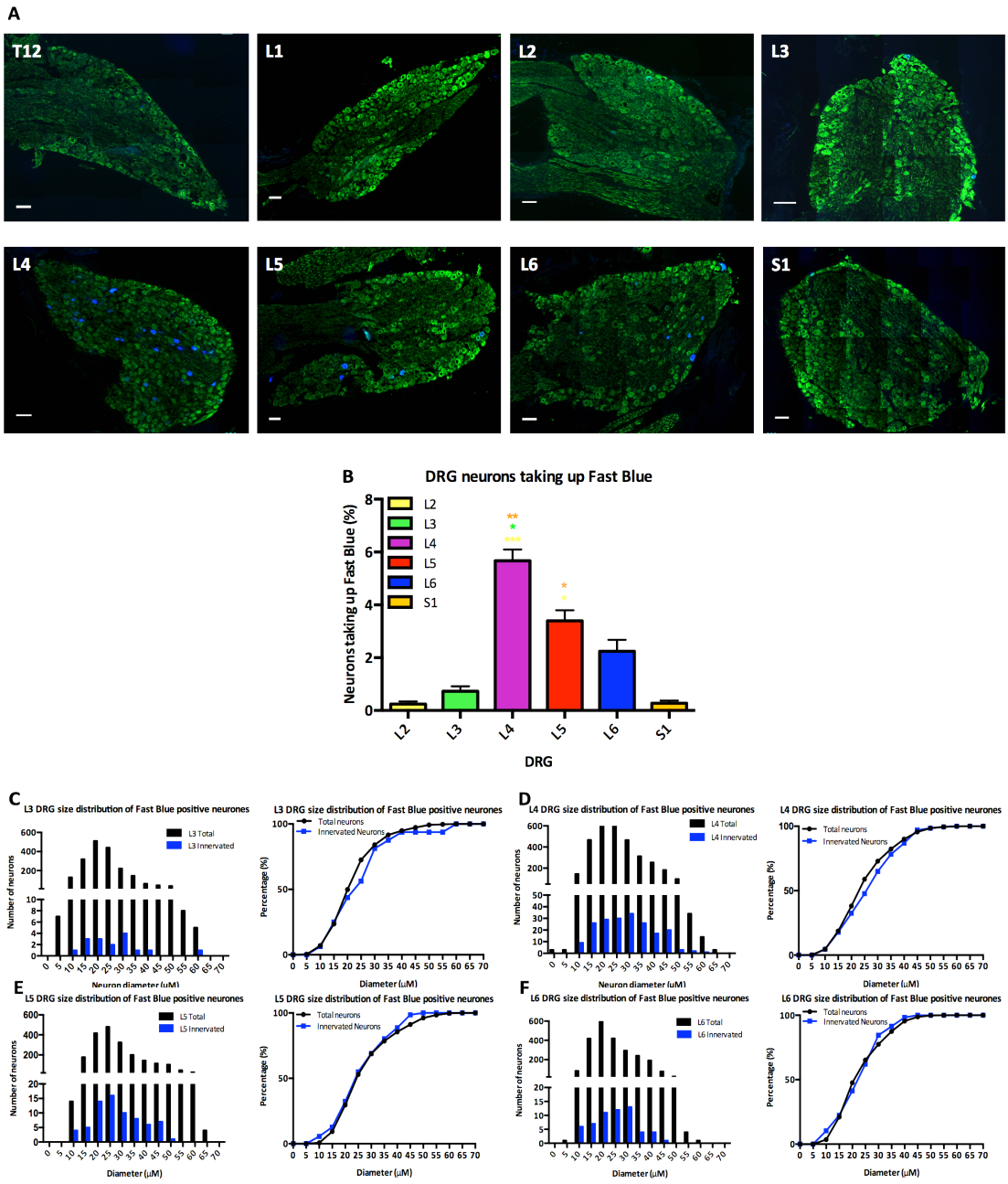


Figure 6.8 Investigation of Fast Blue staining in lumbar DRGs, to assess which peripheral afferent fibres were taking up the neuronal tracer. A) Green staining = βIII-tubulin, Blue staining = Fast Blue neuronal tracer, all scale bars = 100μM. The ipsilateral DRGs from T12–S1 were investigated in animals that had received an intrarticular injection of Fast Blue. Positive Fast Blue staining was identified in L2-S1 DRGs with the most neurons taking up Fast Blue in L4 and L5 DRGs (animal n=3, slice n=12). B) The percentage of total neurons in the ipsilateral DRGs that showed positive staining for Fast Blue, this indicates that the L4 and L5 DRGs had a significantly higher proportion of neurons stained positive for the neuronal tracer compared to the other ipsilateral DRGs (n=3). C) The size distribution of neurons in the ipsilateral L3 DRG taking up the Fast Blue neuronal tracer compared to the size distribution of total neurons in the DRG. D) The size distribution of neurons in the ipsilateral L4 DRG taking up the Fast Blue neuronal tracer compared to the size distribution of total neurons in the DRG. E) The size distribution of neurons in the ipsilateral L5 DRG taking up the Fast Blue neuronal tracer compared to the size distribution of total neurons in the DRG. F) The size distribution of neurons in the ipsilateral L6 DRG taking up the Fast Blue neuronal tracer compared to the size distribution of total neurons in the DRG. Friedmans two-way ANOVA by ranks test was used to

test statistical differences in the proportion of neurons taking up the Fast Blue neuronal tracer between the ipsilateral DRGs. *P<0.05, **P<0.01, ***P<0.001.

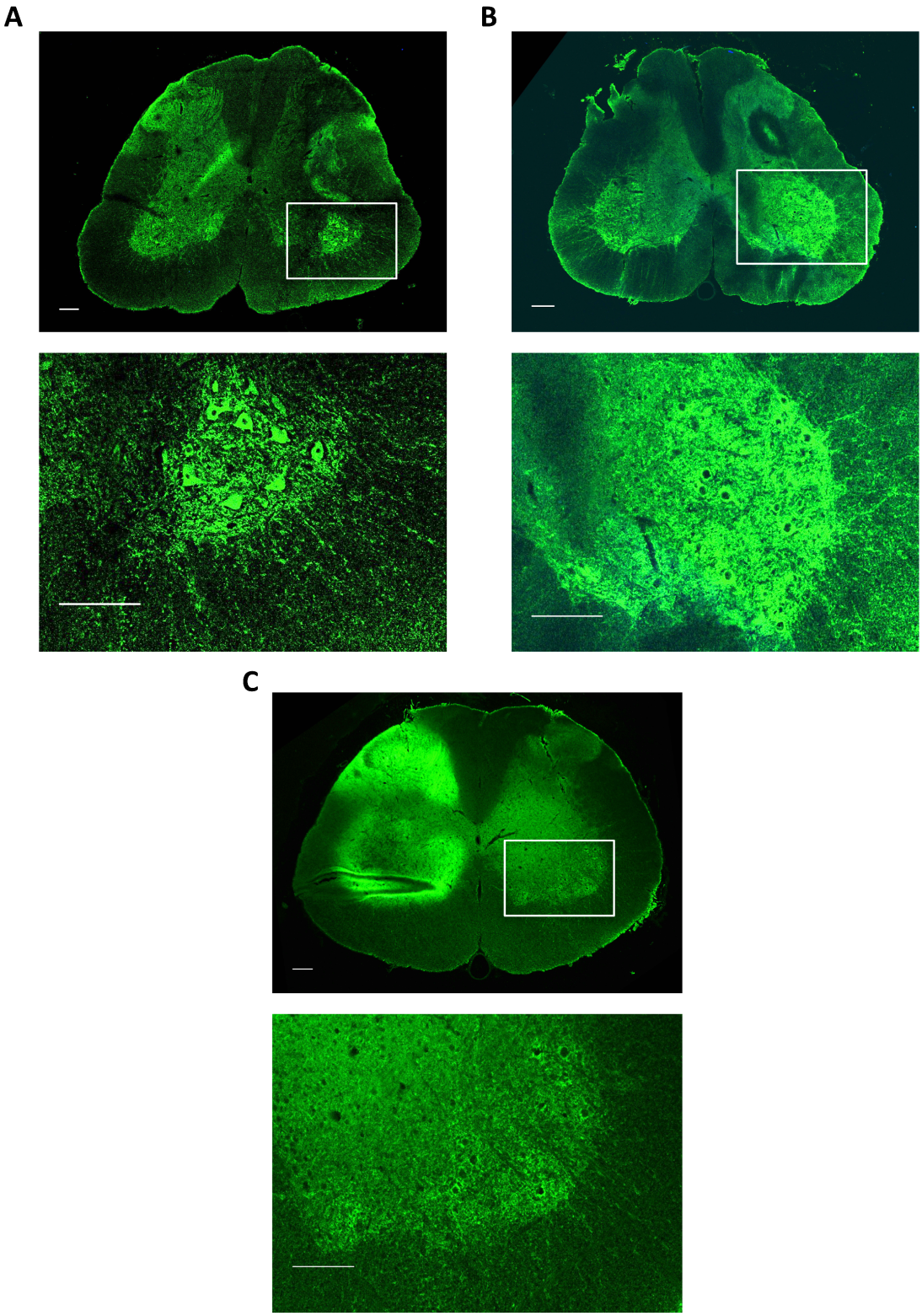


Figure 6.9 Investigation of Fast Blue neuronal tracer staining in motor neurons in the lumbar ventral horn. The lumbar spinal cords were investigated in animals who had received an intrarticular injection of Fast Blue (n=3). Green staining = βIII-tubulin, Blue staining = Fast

Blue neuronal tracer, all scale bars = 500μM. A-C) An example lumbar spinal cord slice from each injected animal, no motor neurons within the ventral horn showed positive Fast Blue staining.

6.3.5 A neuropathic component to the MIA model

6.3.5.1 The efficacy of pregabalin in the MIA model

As the 2mg MIA model has been reported to evoke a neuropathic component both within and surrounding the joint, and pregabalin has proved effective in both patients and animals models with neuropathic pain, the efficacy of pregabalin was investigated in early and late phase MIA animals (Thakur et al 2012).

In early phase MIA animals, pregablin proved ineffective at significantly inhibiting pre-conditioned mechanically evoked neuronal responses and had a limited effect on the level of inhibition induced by DNIC (Figure 6.10A and 6.10B). Before a systemic dose of pregabalin in early phase MIA animals, the levels of mechanically evoked neuronal inhibition induced a concurrent noxious ear pinch were 47%, 38%, and 33% and with a concurrent noxious knee pinch were 59%, 49%, and 40% for 8g, 26g, and 60g stimulations respectively. Following a 10mg/kg systemic dose of pregabalin, the levels of mechanically the levels of mechanically evoked neuronal inhibition induced a concurrent noxious ear pinch were 58%, 33%, and 37% and with a concurrent noxious knee pinch were 45%, 41% and 39% for 8g, 26g, and 60g stimulations respectively. Therefore, the levels of mechanically evoked neuronal inhibition induced by DNIC were of comparative levels both before and after a systemic injection of pregabalin. Similar findings were observed in early phase saline injected sham controls, as pregabalin had a limited effect on the pre-conditioned mechanically evoked neuronal firing or the levels of inhibition induced by DNIC (Figure 6.10C and 6.10D). Overall, these findings indicated that pregabalin was ineffective in early phase MIA and sham animals at reducing mechanically evoked neuronal firing.

In late phase MIA animals, pregabalin significantly inhibited mechanically evoked neuronal responses at all mechanical stimulations tested (Figure 6.10F). However, pregabalin proved ineffective at restoring DNIC in late phase MIA animals, as there was no significant reduction in mechanically evoked neuronal firing with a concurrent noxious ear pinch (Figure 6.10E). Additionally, despite an observed reduction in 26g mechanically evoked neuronal firing with a concurrent noxious knee pinch, there was

no significant reduction in mechanically evoked neuronal firing with a concurrent noxious knee pinch for any mechanical stimulations tested (Figure 6.10E). It should also be noted that a reduction in mechanically evoked neuronal firing with a concurrent noxious knee pinch has been observed in late phase MIA animals before drug application, so a reduction in neuronal firing with a concurrent noxious knee pinch but not a concurrent noxious ear pinch would not necessarily demonstrate a restored DNIC system. The ability of pregabalin to significantly reduce mechanically evoked neuronal firing may be indicative of central sensitization or a neuropathic component of pain in late phase MIA animals, which are sufficient conditions for pregabalin to become efficacious.

In late phase saline injected sham controls, pregabalin did not inhibit pre-conditioned mechanically evoked neuronal firing or affect the level of neuronal inhibition induced with DNIC (Figure 6.10H and 6.10I). The pre-drug neuronal inhibition levels in late phase shams with a concurrent noxious ear pinch were 48%, 38%, and 34% and with a concurrent noxious knee pinch were 53%, 44% and 42% for 8g, 26g and 60g stimulations respectively (Figure 6.10H). Following a 10mg/kg systemic dose of pregabalin, the levels of neuronal inhibition induced by a concurrent noxious ear pinch were 38%, 37% and 33%, and with a concurrent noxious knee pinch were 52%, 33%, and 39% for 8g, 26g and 60g stimulations respectively (Figure 6.10H). Therefore, the levels of neuronal inhibition induced by DNIC were comparable before and after drug administration, indicating pregabalin was having a limited effect on the endogenous inhibitory system in late phase sham animals.

6.3.5.2 ATF-3 expression within DRG cells and ventral horn motor neurons following MIA injection

ATF-3 is a transcription factor, whose expression is upregulated in a variety of tissues in response to stressful stimuli (Hunt et al 2012). ATF-3 expression has been shown to be induced in sensory and motor neurons in the DRGs and spinal cord in sciatic nerve transection and neuronal crush animal models (Tsujino et al 2000, Kataoka et al 2007). ATF-3 was found to be expressed in DRG and spinal cord neurons 3 days following nerve crush or transection and remained up to 84 days post injury, with the number of neurons expressing ATF-3 slowing declining over time (Tsujino et al 2000, Kataoka et al 2007). Therefore, ATF-3 is considered a sensitive marker for detecting neuronal damage or injury.

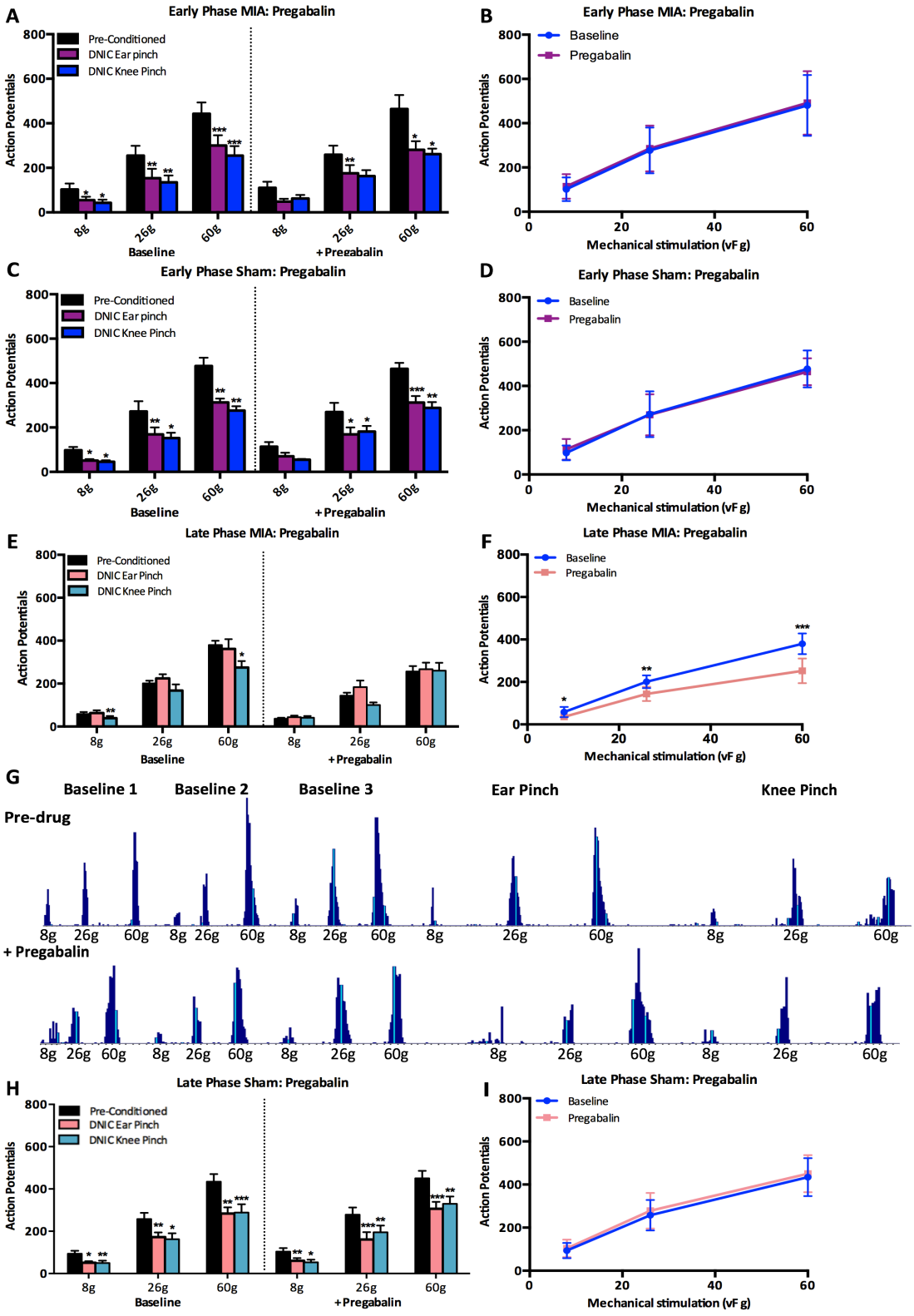


Figure 6.10 The efficacy of pregabalin in the MIA model. A) A 10mg/kg systemic dose of pregabalin did not affect the level of neuronal inhibition induced by DNIC in early phase MIA animals (n=5). B) pregabalin did not inhibit the pre-conditioned mechanically evoked neuronal responses in early phase MIA animals. C) Pregabalin did not affect the level of neuronal inhibition induced by DNIC in early phase sham animals (n=5). D) Pregabalin did not inhibit the pre-conditioned mechanically evoked neuronal responses in early phase sham animals. E) A 10mg/kg systemic dose of pregabalin did not restore DNIC in late phase MIA animals (n=6). F)

Pregabalin significantly inhibited pre-conditioned mechanically evoked neuronal responses. G) An example trace from one WDR neuron showing mechanically evoked responses. The pre-drug DNIC trial indicates no neuronal inhibition was induced with a concurrent noxious ear pinch. The post pregabalin DNIC trial shows lower pre-conditioned neuronal responses but no neuronal inhibition was induced with a concurrent noxious ear pinch. H) Pregabalin did not affect the level of inhibition induced by DNIC in late phase sham animals (n=6). I) Pregabalin did not inhibit the pre-conditioned mechanically evoked neuronal responses in late phase sham animals. Two-way ANOVA with Bonferroni correction. *P<0.05, **P<0.01, ***P<0.001.

The expression of ATF-3 was assessed in DRG cell bodies in early phase and late phase animals following MIA injection, to investigate if the MIA injection was causing any nerve trauma. In contrast to previous studies using the 2mg MIA model, no positive ATF-3 staining was observed in L3-L5 DRGs taken from either early phase animals on day 4, or late phase animals on day 14 (Thakur et al 2012) (Figure 6.11B and 6.11D). Due to the lack of staining, the ATF-3 antibody was tested using DRGs taken from an SNL rat model of neuropathy. ATF-3 expression was observed in the nucleus of lumbar DRG cells taken from the SNL rat, indicating the antibody was functional and binding to ATF-3 (Figure 6.11A). Therefore, the lack of ATF-3 positive neurons in the lumbar DRGs taken from early phase and late phase MIA animals indicates the absence of nerve trauma caused by the MIA injection. There were also no ATF-3 positive cell bodies observed in lumbar DRGs taken from early and late phase saline injected sham controls (Figure 6.11C and 6.11E).

The expression of ATF-3 was also assessed in motor neurons in the ventral horn of the spinal cord, to investigate if the MIA injection was leaking out and damaging motor neurons running adjacent to the knee joint. There was no positive ATF-3 staining identified in lumbar spinal cord slices from either early or late phase MIA animals (Figure 6.12A and 6.12C). This also contrasts with previous findings using the 2mg MIA model, where ATF-3 positive motor neurons were observed in the ventral horn (Thakur et al 2012). The absence of ATF-3 staining in the lumbar spinal cord indicates that it is unlikely the MIA injection is leaking from the intrarticular space and damaging motor neurons surrounding the knee joint. There were also no ATF-3 positive neurons found in the lumbar spinal cords taken from early and late phase saline injected sham controls (Figure 6.12B and 6.12D). The absence of ATF-3 staining in both the lumbar DRG cell bodies and motor neurons in the ventral horn suggests the absence of a neuropathic component to the MIA model.

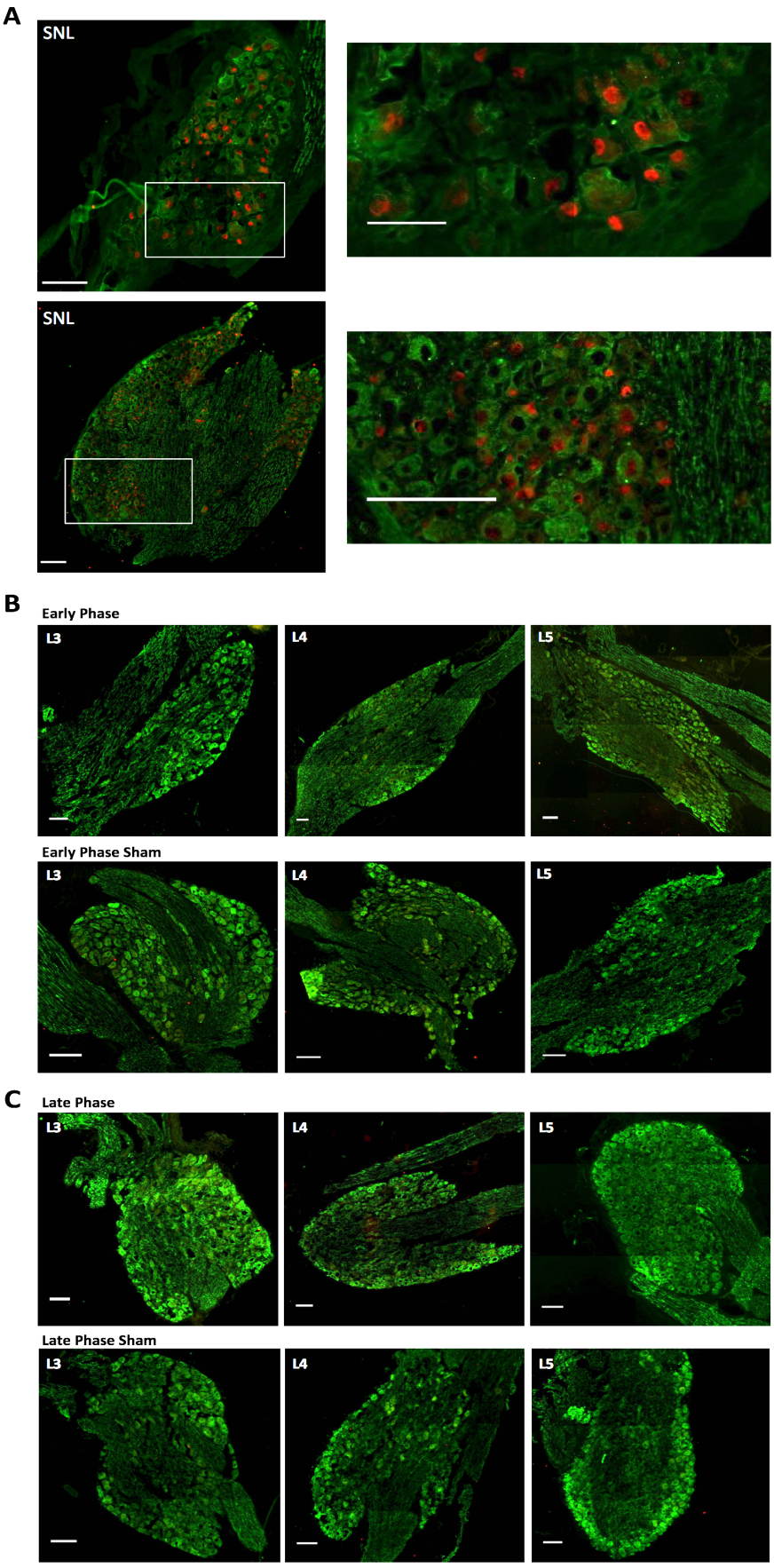


Figure 6.11 The expression of ATF-3 in the cell bodies of lumbar DRGs. Green staining = βIII-tubulin, Red staining = nuclear ATF-3 staining. All scale bars = 100μM A) Lumbar DRGs taken

from rats following spinal nerve ligation show ATF-3 positive cell bodies indicating nerve trauma (n=3). B) There were no ATF-3 positive cell bodies in the lumbar DRGs taken from early phase MIA and sham animals, indicating a lack of nerve injury (EP n=3, EPS n=3). C) There were no ATF-3 positive cell bodies in the lumbar DRGs taken from late phase MIA and sham animals, indicating a lack of nerve injury (LP n=5, LPS n=4).

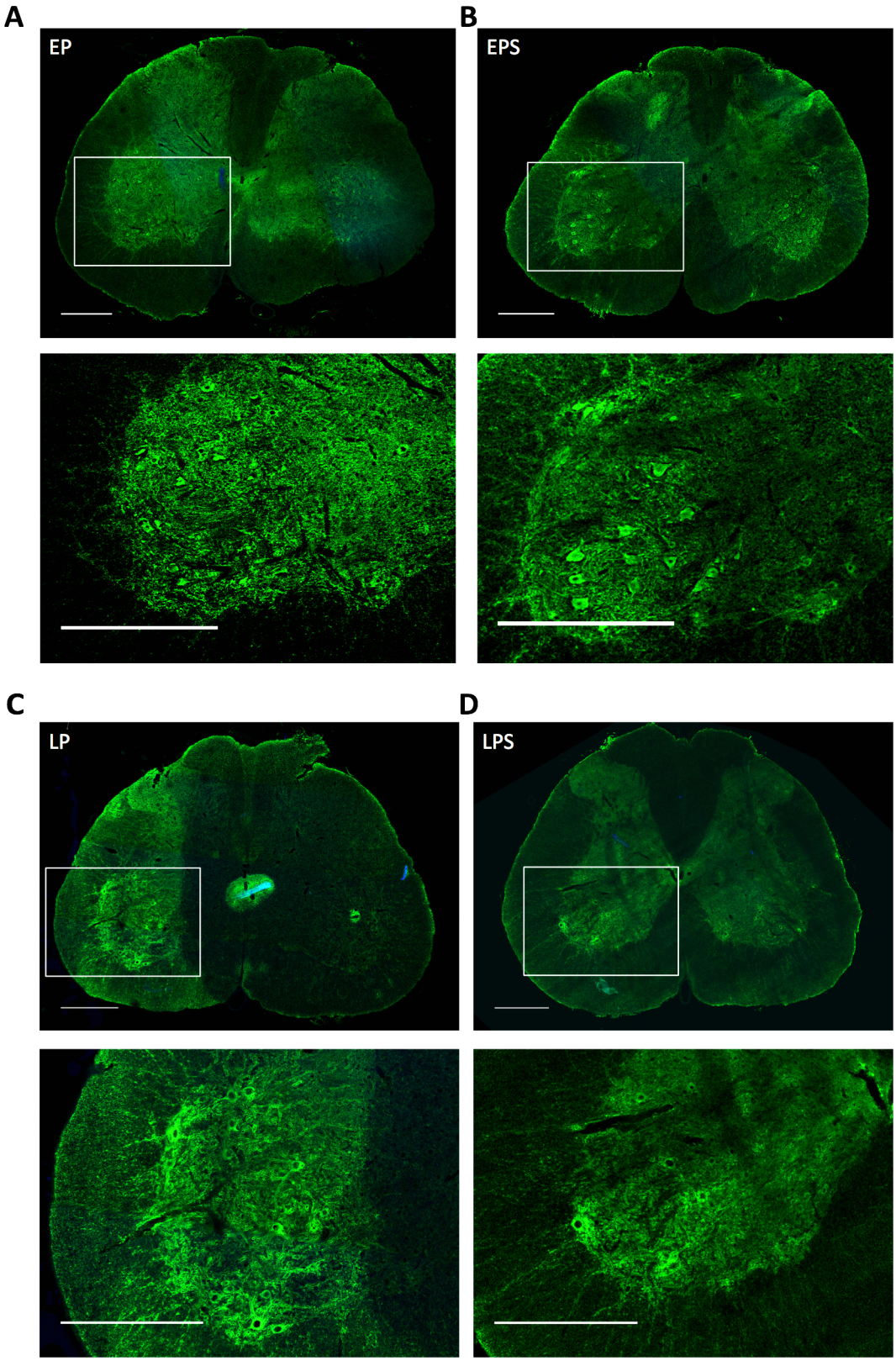


Figure 6.12 The expression of ATF-3 in the motor neurons in the lumbar ventral horn. Green staining = βIII-tubulin, red staining = ATF-3, all scale bars = 500 μM. EP) There was no

ATF-3 positive motor neurons in the lumbar ventral horn taken from early phase MIA animals (n=3). EPS) There was no ATF-3 positive motor neurons in the lumbar ventral horn from early phase saline injected sham controls animals (n=3). LP) There was no ATF-3 positive motor neurons in the lumbar ventral horn from late phase MIA animals (n=5). LPS) There was no ATF-3 positive motor neurons in the lumbar ventral horn from late phase saline injected sham controls (n=4).

6.3.5.3 The mRNA expression of ATF-3 and the $\alpha_2\delta_1$ subunit in lumbar DRGs and dorsal horns from MIA animals

To further explore if there was a neuropathic component associated with the MIA model, the mRNA expression of the nerve injury marker ATF-3 and the $\alpha_2\delta_1$ subunit of VGCCs, which is upregulated and contributes to spinal hyperexcitability in states of neuropathy, was quantified in the ipsilateral lumbar DRGs and dorsal horn following MIA injection (Li et al 2006).

An increased mRNA expression of ATF-3 was observed in the ipsilateral lumbar dorsal horn taken from both early and late phase MIA animals compared to their respective sham control group (Figure 6.13A). For early phase MIA animals, the ATF-3 mRNA expression in the dorsal horn was not significantly higher than the early phase sham control group when the data was normalized to sham or expressed as ΔCT values (Figure 6.13A and 6.13B). In late phase MIA animals the ATF-3 mRNA expression in the dorsal horn was not significantly increased when normalized to the late phase sham group. However, there was a significantly increased ATF-3 mRNA expression in the ipsilateral lumbar dorsal horn when expressed as ΔCT values and compared to late phase saline injected sham controls (Figure 6.13C). Initially, this result was surprising as no positive ATF-3 staining had been identified in the dorsal horn of spinal cord slices in the immunohistochemistry experiment (Figure 6.12). However, microglia are reported to have a high mRNA expression of ATF-3, and as microgliosis has been reported in late phase MIA animals, both in this thesis and in previous studies, it is likely the increased ATF-3 mRNA expression levels reflect increased microglia expression in the dorsal horn (See Section 6.4.3.2)(Thakur et al 2012, Zhang et al 2014).

There was no significant increase in the mRNA expression of ATF-3 in the ipsilateral L3-L5 DRGs in either early or late phase MIA animals compared to saline injected sham controls (Figure 6.13F, 6.13G and 6.13H). This is in agreement with immunohistochemical studies, where no ATF-3 positive cell bodies were identified in the L3-L5 DRGs in either early or late phase MIA animals (Figure 6.11). Overall, the

combination of these findings suggest the 2mg MIA injection is not causing nerve damage to peripheral afferents within and surrounding the knee joint.

Interestingly, there was no increase in the mRNA expression of the $\alpha_2\delta_1$ subunit of VGCCs in the ipsilateral dorsal horn from either early phase or late phase MIA animals compared to their respective sham group (Figure 6.13A, 6.13D and 6.13E). Additionally, there was no increase in the mRNA expression of the $\alpha_2\delta_1$ subunit of VGCCs in the ipsilateral L3-L5 DRGs from early or late phase MIA animals compared to their respective sham group (Figure 6.13F, 6.13I and 6.13J). As there is no increased expression of the $\alpha_2\delta_1$ subunit mRNA in either the lumbar DRGs or dorsal horn of late phase MIA animals, it indicates that the efficacy of Pregabalin in late phase MIA animals is not due to an upregulated expression of the $\alpha_2\delta_1$ subunit and a subsequent increase in trafficking of VGCCs to plasma membranes (Bauer et al 2009).

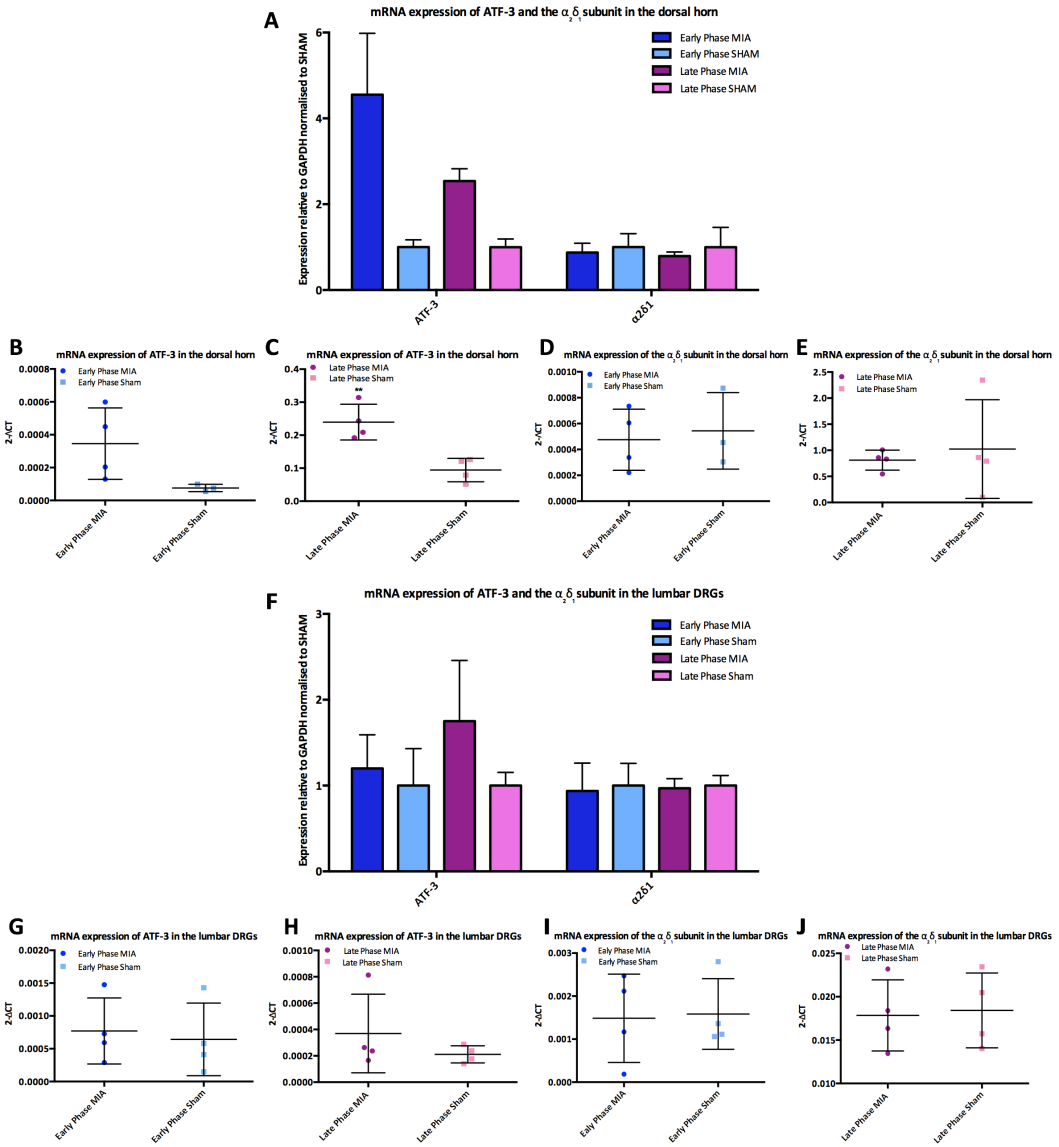


Figure 6.13 The mRNA expression of ATF-3 and the $\alpha_2\delta_1$ subunit in the lumbar dorsal horn and DRGs following MIA injection. A) The mRNA expression of ATF-3 and the $\alpha_2\delta_1$ subunit of VGCCs relative to the housekeeping gene GAPDH in the ipsilateral dorsal horn following MIA injection in early and late phase animals and normalized to their respective sham group (EP n=4, EPS n=3, LP n=4, LPS n=4). B) The mRNA expression of ATF-3 relative to the housekeeping gene GAPDH in the ipsilateral lumbar dorsal horn of early phase MIA and sham animals. C) The mRNA expression of ATF-3 relative to the housekeeping gene GAPDH in the ipsilateral lumbar dorsal horn of late phase MIA and sham animals, this indicates a significantly higher ATF-3 mRNA expression in the dorsal horn of late phase MIA animals compared to saline injected sham controls. D) The mRNA expression of the $\alpha_2\delta_1$ subunit of VGCCs relative to the housekeeping gene GAPDH in the ipsilateral lumbar dorsal horn of early phase MIA and sham animals. E) The mRNA expression of the $\alpha_2\delta_1$ subunit of VGCCs relative to the housekeeping gene GAPDH in the ipsilateral lumbar dorsal horn of late phase MIA and Sham animals. F) The mRNA expression ATF-3 and the $\alpha_2\delta_1$ subunit of VGCCs relative to the housekeeping gene GAPDH in the ipsilateral L3-L5 DRGs following MIA injection in early and late phase animals normalized to their respective sham group (EP n=4, EPS n=4, LP n=4, LPS n=4). G) The mRNA expression of ATF-3 relative to the housekeeping gene GAPDH in the ipsilateral L3-L5 DRGs of early phase MIA and sham animals. H) The mRNA expression of ATF-3 relative to the housekeeping gene GAPDH in the ipsilateral L3-L5 DRGs of late phase MIA and sham animals. I) The mRNA expression of the $\alpha_2\delta_1$ subunit of VGCCs relative to the housekeeping gene GAPDH in the ipsilateral L3-L5 DRGs of early phase MIA and sham animals. J) The mRNA expression of the $\alpha_2\delta_1$ subunit of VGCCs relative to the housekeeping gene GAPDH in the ipsilateral L3-L5 DRGs of early phase MIA and sham animals. For mRNA expression values normalized to sham controls, statistical significance between MIA and sham groups was tested with a Kruskall-wallis independent samples one-way ANOVA. For mRNA expression expressed as ΔCT values, statistical significance between MIA and sham groups was tested with independent samples T-tests. **P<0.01.

6.3.6 The combination of tapentadol and pregabalin treatment in MIA animals

Tapentadol was demonstrated to dose-dependently inhibit pre-conditioned mechanically evoked neuronal responses and restore neuronal inhibition induced by DNIC in late phase MIA animals (Figure 6.5). However, the lower doses of tapentadol tested were only effective at reducing pre-conditioned mechanically evoked neuronal responses to the most noxious stimulation tested of 60g. As a low dose of tapentadol did not inhibit pre-conditioned neuronal responses to innocuous stimulations, this may indicate that low doses of tapentadol would be ineffective at relieving allodynia (Figure 6.5B). Meanwhile, pregabalin proved effective at inhibiting pre-conditioned mechanically evoked neuronal responses to all stimulations tested in late phase MIA animals but did not restore neuronal inhibition induced by DNIC (6.10). Therefore, a combination therapy of tapentadol and pregabalin was assessed in late phase MIA and sham animals to investigate if this may be an effective approach for inhibiting pre-conditioned neuronal responses, whilst also restoring neuronal inhibition induced by DNIC.

In late phase MIA animals, the combined tapentadol and pregabalin treatments restored neuronal inhibition induced by DNIC and significantly inhibited pre-conditioned

mechanically evoked neuronal responses for all mechanical stimulations tested (Figure 6.14A-D). Firstly, a combined systemic dose of 1mg/kg tapentadol and 10mg/kg of pregabalin restored neuronal inhibition such that there was a significant reduction in neuronal firing with a concurrent noxious ear and knee pinch and mechanical stimulations of 26g and 60g (Figure 6.14A). The level of neuronal inhibition following a systemic dose of 1mg/kg tapentadol and 10mg/kg pregabalin was 41%, 34%, and 26% when induced with a noxious ear pinch and 52%, 29% and 33% when induced with a noxious knee pinch for 8g, 26g, and 60g mechanical stimulations respectively, which is approximately the level of neuronal inhibition observed in naïve animals with a conditioning noxious stimulus. Secondly, a combined systemic dose of 2mg/kg tapentadol and 5mg/kg of pregabalin also restored neuronal inhibition such that there was a significant reduction in neuronal firing with a concurrent noxious ear and knee pinch for 26g and 60g mechanical stimulations (Figure 6.14B). The level of neuronal inhibition following 2mg/kg tapentadol and 5mg/kg pregabalin was also of a similar level to that observed in naïve animals with a concurrent noxious stimulation. Finally, both dose combinations significantly inhibited pre-conditioned evoked neuronal firing for all mechanical stimulations tested, indicating a combined dose is more effective at inhibiting neuronal firing than a dose of tapentadol alone.

In late phase saline injected sham control animals, both dose combinations had a limited effect on the levels of neuronal inhibition induced by DNIC (Figure 6.14E and 6.14G). Interestingly, the combined systemic dose 1mg/kg tapentadol and 10mg/kg pregabalin did not significantly inhibit pre-conditioned mechanically evoked neuronal firing for any mechanical stimulations tested (Figure 6.14F). This is in agreement with previous experiments demonstrating that a 10mg/kg dose of pregabalin has no inhibitory effect on neuronal firing in saline injected sham controls. This may indicate that without sufficient conditions for pregabalin to be effective, the low dose of tapentadol alone is not enough to inhibit pre-conditioned neuronal firing. However, the combined 2mg/kg tapentadol and 5mg/kg pregabalin dose does significantly inhibit the pre-conditioned neuronal firing for the 60g mechanical stimulation (Figure 6.14H). This indicates that although pregabalin is not effective in sham controls, the higher dose of tapentadol is sufficient to inhibit neuronal firing to the most noxious mechanical stimulations.

Overall, there is a lack of inhibitory effect on pre-conditioned mechanically evoked neuronal firing of tapentadol and pregabalin combined therapy in sham control animals. However, the combined tapentadol and pregabalin treatment proved effective for

inhibiting pre-conditioned neuronal firing for all mechanical stimulations tested in late phase MIA animals. This suggests that with sufficient conditions, such as central sensitisation and an imbalance of descending controls, the combined therapy with lower doses of tapentadol and pregabalin is most effective at producing both pre-conditioned neuronal inhibition and restoring the DNIC system.

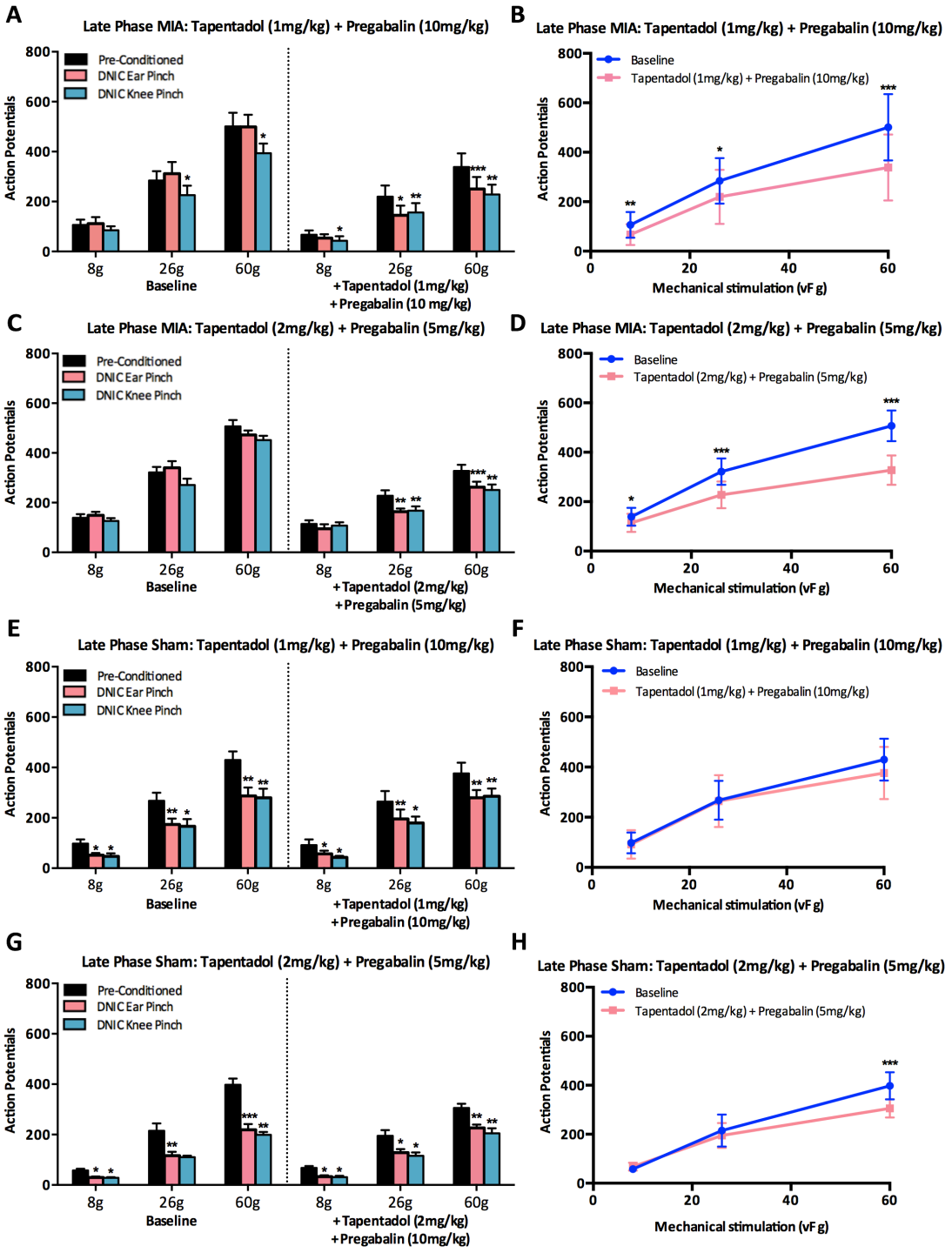


Figure 6.14 A combined tapentadol and pregabalin treatment in MIA animals. A) A systemic dose of 1mg/kg tapentadol and 10mg/kg pregabalin restores neuronal inhibition induced with a

concurrent noxious ear and knee pinch in late phase MIA animals (n=6). B) A systemic dose of 1mg/kg tapentadol and 10mg/kg pregabalin significantly inhibits pre-conditioned mechanically evoked neuronal firing in late phase MIA animals. C) A systemic dose of 2mg/kg tapentadol and 5mg/kg pregabalin restores neuronal inhibition induced with a concurrent noxious ear and knee pinch in late phase MIA animals (n=6). D) A systemic dose of 2mg/kg tapentadol and 5mg/kg pregabalin significantly inhibits pre-conditioned mechanically evoked neuronal firing in late phase MIA animals. E) A systemic dose of 1mg/kg tapentadol and 10mg/kg pregabalin has a limited effect on the level of neuronal inhibition induced by DNIC in late phase sham animals (n=6). F) A systemic dose of 1mg/kg tapentadol and 10mg/kg pregabalin has no significant effect on pre-conditioned mechanically evoked neuronal firing in late phase sham animals. G) A systemic dose of 2mg/kg tapentadol and 5mg/kg pregabalin has a limited effect on the level of neuronal inhibition induced by DNIC in late phase sham animals (n=5). H) A systemic dose of 2mg/kg tapentadol and 5mg/kg pregabalin significantly inhibited pre-conditioned neuronal firing in response to a 60g mechanical stimulation. Two-way ANOVA with Bonferroni correction. *P<0.05, **P<0.01, ***P<0.001.

6.4 Discussion

This study examined the pharmacological manipulation of DNIC in early and late phase MIA animals. This study indicated that blocking the actions of spinal 5-HT_7 receptors in early phase MIA animals was not sufficient to block DNIC, yet when the 5-HT_7 receptor was activated in late phase MIA animals DNIC was restored (Figure 6.15). In contrast, blocking the inhibitory actions of noradrenaline at α_2-adrenoceptors in the spinal cord completely blocked DNIC in early phase MIA animals (Figure 6.15). These findings are in agreement with those observed in chapter 4, that the inhibitory actions of noradrenaline are crucial for the expression of DNIC, while serotonergic controls contribute to the DNIC system but are not crucial. Yet when the DNIC system is lost due to an imbalance in descending controls, as is the case in late phase MIA animals, the activation of spinal 5-HT_7 receptors is enough to override this imbalance and restore inhibition. A systemic dose of Tapentadol also restores DNIC in late phase MIA animals, likely due to its NRI actions meaning more synaptic noradrenaline is available to carry out inhibitory actions at α_2-adrenoceptors.

This study also examined if there was a neuropathic component to the MIA model. While Pregabalin proved ineffective in early phase and sham animals, it was effective at inhibiting mechanically evoked neuronal responses in late phase MIA animals. However, Pregabalin did not affect the magnitude of neuronal inhibition induced by DNIC in either early or late phase MIA animals (Figure 6.15). The sensitivity to Pregabalin may indicate a neuropathic component exists in late phase MIA animals, or that there are sufficient conditions such as central sensitisation or enhanced descending facilitation so that Pregabalin can prove effective. However, there was a lack of ATF-3 staining in the ipsilateral lumbar DRG, which had been demonstrated to contain the cell bodies of peripheral afferents innervating the knee with the use of a neuronal tracer. The absence of ATF-3 expression in late phase animals suggests the MIA injection is not causing damage to nerves within and surrounding the knee joint. Furthermore, there was no upregulated expression of the $\alpha_2\delta_1$ subunit of VGCCs in the lumbar DRGs or dorsal horn indicating Pregabalin was not functioning through this mechanism. Overall, this study indicated that there was sufficient central sensitisation in late phase MIA animals for Pregabalin to prove effective, but there was not necessarily a neuropathic component contributing to the chronic pain associated with the late phase MIA model.

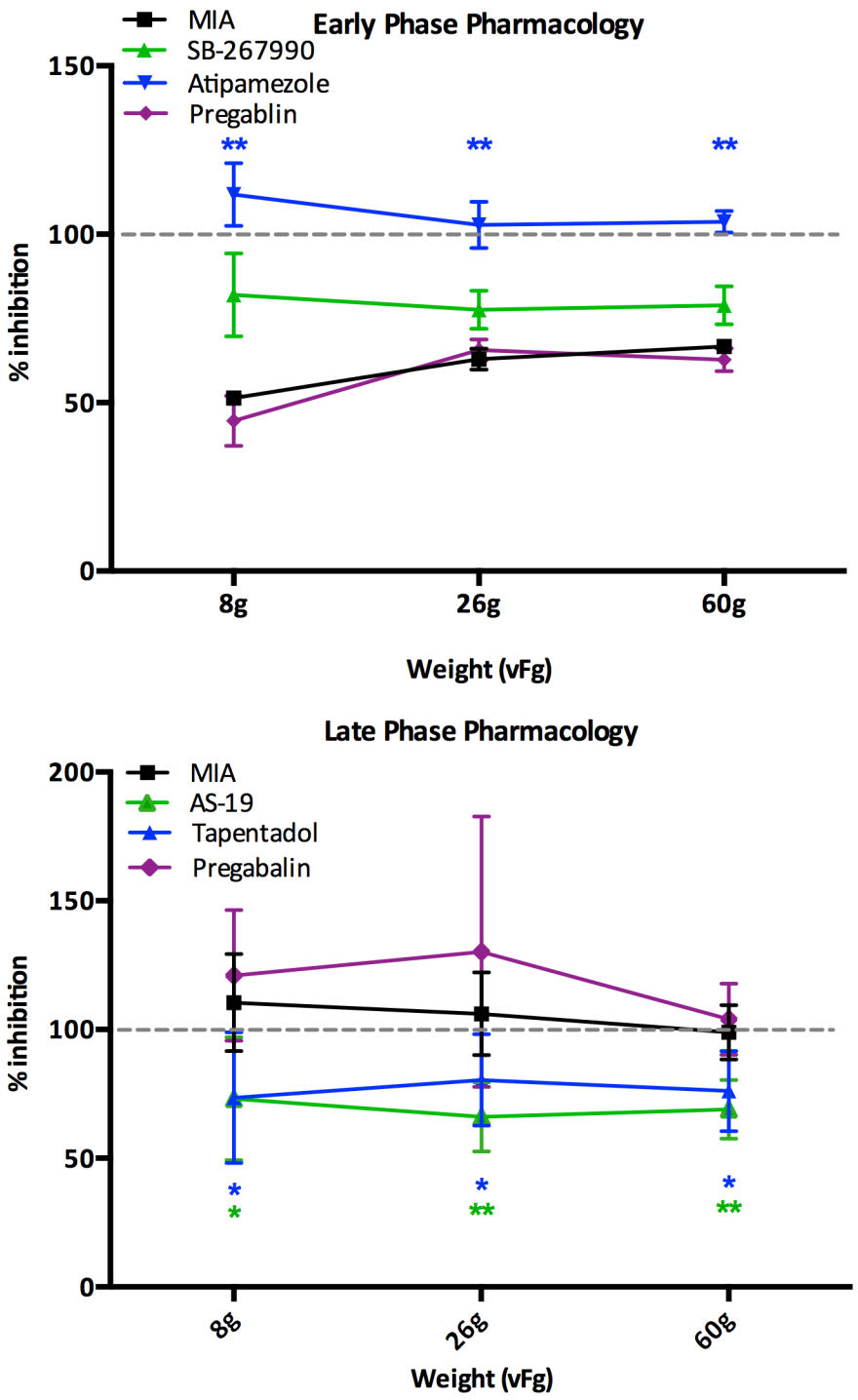


Figure 6.15 The magnitude of neuronal inhibition induced by DNIC in early and late phase MIA animals following drug application. In early phase MIA animals the spinal application of atipamezole abolishes any neuronal inhibition induced by DNIC, while the spinal application of SB-267990 partially blocks the neuronal inhibition induced by DNIC, and systemic pregabalin has no effect on the magnitude of neuronal inhibition induced by DNIC. In late phase MIA animals. systemic tapentadol and spinal AS-19 significantly restore neuronal inhibition induced by DNIC, while systemic pregabalin has no effect on the magnitude of neuronal inhibition induced by DNIC. Kruskall-Wallis one-way ANOVA *P<0.05, **P<0.01.

6.4.1 The noradrenergic and serotonergic descending controls subserving DNIC

6.4.1.1 The 5-HT$_7$ receptor

Firstly, the contribution of spinal 5-HT$_7$ receptors in modulating the endogenous inhibitory system utilized by DNIC was explored in early phase MIA animals, where a functional DNIC system existed. While the selective 5-HT$_7$ receptor antagonist SB-267790 reduced the level of neuronal inhibition induced with a concurrent noxious ear or knee pinch it did not abolish the DNIC system in early phase MIA and sham animals. The 5-HT$_7$ receptor has been demonstrated to play an inhibitory role in uninjured animals, as blocking the actions of spinal 5-HT$_7$ receptors blocked the antinociceptive effects observed following both a systemic or RVM microinjection of morphine (Dogrul and Seyrek 2006, Dogrul et al 2009). In this case the selective 5-HT$_7$ receptor antagonist SB-267790 completely blocked antinociception, which indicates a principle role for this receptor in mediating opioidergic controls arising from the RVM, yet the DNIC system is reported to rely only partly on opioids (Le Bars et al 1981). Overall, this study indicated that while the 5-HT$_7$ receptor contributes to the neuronal inhibition induced by DNIC, blocking these actions cannot abolish the endogenous inhibitory system, therefore inhibitory actions occurring through the activation of other spinal receptors are still able to mediate the inhibitory DNIC response.

The activation of 5-HT$_7$ receptors has been demonstrated to mediate antinociceptive actions in animal models of nerve injury and inflammation (Brenchat et al 2009, Brenchat et al 2010, Lin et al 2015). Furthermore, the 5-HT$_7$ receptor is co-localized with GABAergic interneurons in the dorsal horn, and has been demonstrated to mediate its inhibitory actions through a GABAergic component rather than an opiodergic mechanism following nerve injury (Viguier et al 2012). Therefore, the role played by spinal 5-HT$_7$ receptors in modulating endogenous inhibition was assessed in late phase MIA animals, where the DNIC system was abolished. The activation of spinal 5-HT$_7$ receptors with the selective agonist AS-19 restored neuronal inhibition induced with a concurrent noxious ear or knee pinch, indicating that activation of spinal 5-HT$_7$ receptors was sufficient to override the imbalance in inhibitory and facilitatory descending controls and mediate inhibition. Interestingly, the acute administration of 5-HT$_7$ receptor agonists was demonstrated to reduce the nerve injury induced upregulated mRNA expression of IL-1β in the lumbar DRGs and spinal cord, proposing this may be a further mechanism in which 5-HT$_7$ receptors mediate hyperalgesic effects (Viguier et al 2012). In agreement, an increased mRNA expression of IL-1β was found in

the lumbar dorsal horn of late phase MIA animals (See section 5.3.5), and could be related to the inhibitory actions observed following activation of spinal 5-HT_7 receptors. Overall, this study confirmed an inhibitory role for spinal 5-HT_7 receptors, particularly in states of central sensitisation.

6.4.1.2 Noradrenalin acting at α_2-adrenoceptors in the spinal cord

The role played by noradrenaline acting at α_2-adrenoceptors in the spinal cord in mediating DNIC was assessed in early phase MIA animals, where a functional DNIC system remained. Blocking the actions of spinal α_2-adrenoceptors completely blocked the neuronal inhibition induced by a concurrent noxious ear and knee pinch in early phase MIA and sham animals. This indicates that the inhibitory actions of noradrenaline acting at α_2-adrenoceptors in the spinal cord are crucial for DNIC expression. This is in agreement with previous studies, which indicate that even with enhanced inhibitory serotonergic actions that restore DNIC in nerve injured animals, a tonic noradrenergic tone, even at reduced levels is required for DNIC expression (Bannister et al 2017).

Interestingly, blocking the actions of noradrenaline acting at α_2-adrenoceptors in the spinal cord significantly facilitated mechanically evoked neuronal responses in early phase MIA animals but did not have this effect in sham controls. This may be due to enhanced noradrenergic inhibitory descending controls, as have been previously reported in the early inflammatory phase of the MIA model (Burnham and Dickenson 2013). Overall, as noradrenergic signaling through α_2-adrenoceptors are crucial for mediating neuronal inhibition induced by a noxious conditioning stimulation, variations in the noradrenergic descending controls over the course of the MIA model appear to play a principle role in the subsequent expression of DNIC.

6.4.1.3 The mRNA expression of noradrenergic and serotonergic receptors in the lumbar DRGs and dorsal horn

This study indicated the important roles played by α_2-adrenoceptors and 5-HT_7 receptors in mediating neuronal inhibition induced by DNIC. In addition, enhanced serotonergic descending facilitatory controls have been reported to be involved with abolished DNIC in animal models of chronic pain (Bannister et al 2015). In a chronic constriction injury model of neuropathic pain, the mRNA expression of the α_2-

adrenoceptor was found to be reduced in the lumbar spinal cord compared with controls rats (Leiphart et al 2003). Furthermore, an increase in the 5-HT_7 receptor density has been reported in the ipsilateral dorsal horn following partial sciatic nerve ligation, which lead to the authors proposing a pain-triggered regulated expression (Brenchat et al 2010). Together, these findings indicate that changes in the regulation of noradrenergic and serotonergic receptor expression may be contributing to the development of central sensitisation. Therefore, the mRNA expression of these receptors was assessed in the lumbar DRGs and dorsal horn to investigate if an up or down regulation of receptor expression was contributing to the abolished DNIC system in late phase MIA animals.

In fact, this study found that there were no changes in the mRNA expression of the α_2-adrenoceptors, 5-HT_3 receptors or 5-HT_7 receptors in the lumbar DRGs or dorsal horn. These results indicate that the imbalance in inhibitory and facilitatory descending controls is not necessarily a consequence of an altered receptor expression in the spinal cord. As the monoaminergic descending controls modulate nociceptive transmission at the level of the spinal cord mainly through volume transmission, the most likely mechanism for the imbalance in descending controls is a decreased release of noradrenaline and an increased release of serotonin from the brainstem (Todd 2010). However, although this study indicates there are no changes in the mRNA receptor expression, other post-translational changes may occur and influence the binding affinity or down-stream signaling of receptors at the level of the spinal cord.

6.4.1.4 The efficacy of tapentadol in the late phase MIA model

Tapentadol has a dual mechanism of action, acting as both an NRI and a MOR agonist. As both noradrenergic and opiodergic components are believed to be involved in the neuronal inhibition induced by DNIC, the efficacy of tapentadol at restoring DNIC in late phase MIA animals was assessed (Le Bars et al 1981). All doses of tapentadol tested restored neuronal inhibition induced by a noxious conditioning ear or knee pinch, whereas tapentadol had a limited effect on the levels of neuronal inhibition induced by DNIC in sham control animals. In addition, the higher doses of tapentadol significantly inhibited pre-conditioned mechanically evoked neuronal firing rates in both late phase MIA and saline injected sham controls animals.

The MOR and NRI contributions to nociceptive modulation of tapentadol at the level of the spinal cord have previously been assessed in a spinal nerve ligation rat model of

neuropathy (Tzschentke et al 2007, Schroder et al 2010, Bee et al 2011). These studies indicated that the inhibitory actions of tapentadol were most effectively blocked by the MOR antagonist naloxone in sham controls animals, while in SNL animals the analgesic efficacy of tapentadol was strongly reduced by the α_2-adrenoceptor antagonist yohimbine or atipamezole, but only moderately reduced by naloxone (Tzschentke et al 2007, Schroder et al 2010, Bee et al 2011). The authors proposed that tapentadol utilizes a predominant opioid mechanism to mediate inhibition in control animals, which shifts to a predominant noradrenergic inhibitory mechanism following central sensitisation. Therefore, the efficacy of tapentadol at inhibiting pre-conditioned mechanically evoked neuronal firing in sham animals is likely due to a predominant opioid mechanism. Meanwhile, the efficacy of tapentadol at restoring neuronal inhibition induced by DNIC in late phase MIA animals, even at low doses, is likely due to its ability to increase the synaptic content of noradrenaline, which can subsequently carry out an inhibitory role at the level of the spinal cord through activating α_2-adrenoceptors.

Interestingly, at all doses tested tapentadol restored neuronal inhibition induced by DNIC for just the noxious mechanical stimulations of 26g and 60g, yet did not restore neuronal inhibition induced by DNIC with the innocuous mechanical stimulation of 8g. This is in agreement with previous findings that demonstrated tapentadol restored neuronal inhibition only for noxious mechanical stimulations following nerve injury. This led the authors to theorize that the opioidergic mechanisms may preferentially act on noxious stimulations, which when combined with the increased noradrenergic content allows tapentadol to restore DNIC (Bannister et al 2015).

6.4.2 A neuropathic component to the MIA model

6.4.2.1 The efficacy of pregabalin in the MIA model

The gabapentinoid drugs are considered key therapies for relieving the chronic pain associated with neuropathy. Previous studies have demonstrated that gabapentinoid drugs are more effective at modulating spinal nociceptive transmission in animal models of nerve injury than those with normal physiological conditions (Bee and Dickenson 2008, Bannister et al 2017). Furthermore, gabapentinoid treatments provide effective pain relief in patients with neuropathy (Moore et al 2014b). As a neuropathic component to the 2mg MIA model have previously been reported, the efficacy of pregablin in early and late phase MIA animals was assessed and its subsequent effect on

the DNIC system was assessed (Ivanavicius et al 2007, Orita et al 2011, Thakur et al 2012).

Pregabalin proved effective in late phase MIA animals, as it significantly inhibited all pre-conditioned mechanically evoked neuronal responses. However, pregablin was ineffective in early phase MIA animals and sham controls. In animal models of neuropathy, there are increased levels of the $\alpha_2\delta_1$ subunit of VGCCs in the ipsilateral lumbar DRGs and dorsal horn (Luo et al 2001, Newton et al 2001, Bauer et al 2009). The increased $\alpha_2\delta_1$ subunit expression, results in increased trafficking of VGCCs to neuronal plasma membrane, which subsequently increases calcium infux and neurotransmitter release (Hendrich et al 2008, Bauer et al 2009). As pregabalin binds the $\alpha_2\delta_1$ subunit with high affinity, a proposed mechanism for the antinociceptive function of pregabalin is thought to occur through its binding to the $\alpha_2\delta_1$ subunit, which subsequently inhibits the enhanced release of neurotransmitters, but the exact mechanism is yet to be determined (Bauer et al 2009).

In this study, there was no increase in the mRNA expression of the $\alpha_2\delta_1$ subunit of VGCCs in the ipsilateral lumbar DRGs or dorsal horn of late phase MIA animals. This result conflicts with a previous MIA study, which demonstrated a significant increase in the mRNA expression of the $\alpha_2\delta_1$ subunit in the L3 and L4 DRGs ipsilateral to MIA injection (Rahman et al 2009). However, this study also found no significant increase in the mRNA expression of the $\alpha_2\delta_1$ subunit in the L5 and L6 DRGs ipsilateral to MIA injection (Figure 6.16). Therefore, as this study investigated mRNA expression pooled from L3-L5 DRGs, it may have missed a subtle increase in the mRNA expression of the $\alpha_2\delta_1$ subunit, especially if this only occurred in one particular lumbar DRG. However, as the mRNA expression levels of the $\alpha_2\delta_1$ subunit remained fairly constant between MIA and sham control animals in this study, it may indicate that the differences observed are due to injection techniques and a difference in the subsequent neuropathic component of the MIA model.

It has been demonstrated that an increased expression of the $\alpha_2\delta_1$ subunit is not necessarily required for pregabalin to prove effective (Suzuki et al 2005, Bannister et al 2011). In fact, the developments of central sensitisation and an enhanced serotonergic facilitatory descending system in animal models of chronic pain have proved sufficient conditions for gabapentinoid drugs to have an antinociceptive action (Suzuki et al 2005). An abolished DNIC system in the late phase MIA animals strongly suggests that

an enhanced serotonergic facilitatory descending system acting at 5-HT_3 receptors in the spinal cord exists (Bannister et al 2015). Overall, the efficacy of pregabalin but lack of an upregulated expression of the $\alpha_2\delta_1$ subunit, indicates sufficient central sensitisation and enhanced descending serotonergic facilitation are sufficient conditions for pregabalin to prove effective at inhibiting neuronal responses in late phase MIA animals.

Despite pregabalin proving effective at inhibiting pre-conditioned mechanically evoked neuronal responses, it did not restore the DNIC system in late phase MIA animals. Although this study suggests that pregabalin becomes effective in late phase MIA animals due to an enhanced descending facilitatory system, pregabalins sole target remains the $\alpha_2\delta_1$ subunit (Davies et al 2007). Therefore, although there is no upregulation of the $\alpha_2\delta_1$ subunit, pregabalin still mediates inhibition of nociceptive transmission in the spinal cord through its actions at this target. Overall, there is no evidence to indicate that pregabalin restores the balance in facilitatory and inhibitory descending controls, and therefore cannot reinstate the DNIC system in late phase MIA animals.

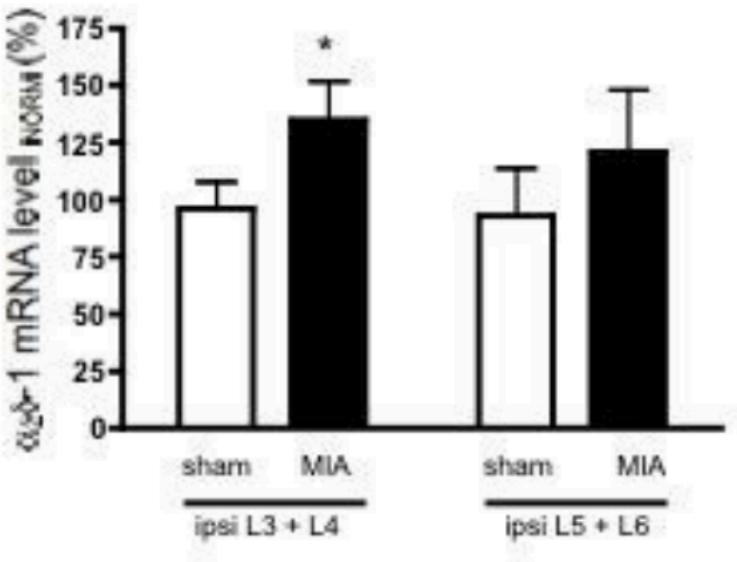


Figure 6.16 The mRNA expression of the $\alpha_2\delta_1$ subunit of VGCCs in lumbar DRGs following MIA injection. The mRNA expression of the α2δ-1 subunit of VGCCs in pooled ipsilateral L3/L4 DRGs or pooled ipsilateral L5/L6 DRGs from sham and MIA animals 14 days post-injection.

6.4.2.2 The expression of ATF-3 in the lumbar DRGs and spinal cord of MIA animals

While the cartilage is not innervated, histology studies indicated that in areas of extreme cartilage degradation there was exposure of the subchondral bone, which is highly innervated, indicating that this extent of damage may lead to peripheral nerve damage (See Section 5.3.2). Retrograde labeling studies have demonstrated that knee joint afferents are localized mainly to L4 DRGs, and the majority of subchondral bone afferents are localized in the L3 DRGs (Aso et al 2014). This was similar to the Fast Blue experiments carried out in this study, which indicated the majority of knee joint afferents were localized in the L4 and L5 DRGs. Therefore, ATF-3 expression was investigated in the L3-L5 DRGs to investigate any nerve trauma in knee joint or subchondral bone afferents.

Remarkably, there was no increase in ATF-3 expression in the ipsilateral L3-L5 DRGs in either early or late phase MIA animals compared to sham controls. This contrasts with previous studies finding a large ATF-3 expression in the lumbar DRGs following a 1mg or 2mg MIA injection, although the exact results vary between studies (Ivanavicius et al 2007, Orita et al 2011, Thakur et al 2012). Firstly, Ivanavicius et al found a significant increase in ATF-3 expression in ipsilateral L4 and L5 DRGs 8 and 14 days following MIA injection but that this expression then decreased and was not significantly higher than sham controls on day 21, 28 and 35 (Ivanavicius et al 2007) Secondly, Orita et al found that ATF-3 positive neurons were only observed in the ipsilateral L4 DRG 14 days following a 2mg MIA injection, but the proportion of ATF-3 positive neurons increased as the model progressed (Orita et al 2011). Finally, Thakur et al found a significant increase in ATF-3 positive neurons in ipsilateral L4 and L5 DRGs as soon as 3 days following MIA injection (Thakur et al 2012).

Interestingly, none of the above studies indicated a significant increase in ATF-3 positive neurons in the L3 DRGs, which indicates the reported neuropathic component was not a result of damage to peripherall afferents innervating the subchondral bone. Firstly, the differences in the ATF-3 staining presented in this thesis and previous findings are not due to differences in MIA dose, as both Thakur et al and Orita et al used a 2mg dose of MIA, while Ivanavicius et al used a 1mg dose, therefore it cannot be that higher MIA doses were causing nerve trauma. Secondly, MIA was diluted in the same volume of saline (25µl) for intrarticular injection in this study and previous studies, indicating that the differing results are not down to the volumes injected (Orita et al 2011, Thakur et al

2012). Thakur et al reported that 20% of L5 DRG cells bodies showed positive staining for ATF-3, whereas the neuronal tracer experiment in this study indicated that only 4%-6% of L4 and L5 neurons innervated the knee (Figure 6.17A)(See Section 6.3.4). Interestingly, a study investigating an MIA mouse model found a significant increase in ATF-3 positive cell bodies in L3-L5 DRGs 7 days after MIA injection, but this was at much lower levels as only 2.1% of cell bodies displayed positive staining (Figure 6.17B)(Ogbonna et al 2013). The authors concluded there was no evidence to suggest MIA induced sensory neuron damage (Ogbonna et al 2013). Overall, the lack of ATF-3 positive cell bodies in L3 DRGs, the high numbers of ATF-3 positive neurons reported, and the presence of ATF-3 positive motor neurons in the ventral horn, suggest primary afferent damage is occurring due to damage outside of the joint and could be attributed to intrarticular injection technique.

The investigation of ATF-3 mRNA expression in the lumbar DRGs confirmed the immunohistochemical findings of this study, as there was no significant increase in the mRNA ATF-3 expression in either early or late phase MIA animals compared to sham controls.

In addition to the ipsilateral lumbar DRGs, the expression of ATF-3 was also assessed in the lumbar spinal cord. Previous MIA studies have also found an expression of ATF-3 in the ventral horn, indicating that ATF-3 expression observed in lumbar DRGs may actually be a result of damage to motor neurons surrounding the knee rather than sensory neurons innervating the knee (Thakur et al 2012). In this study, no ATF-3 expression or Fast Blue staining was observed in the ventral horn, which may indicate a more precise intrarticular injection or less leakage in this study.

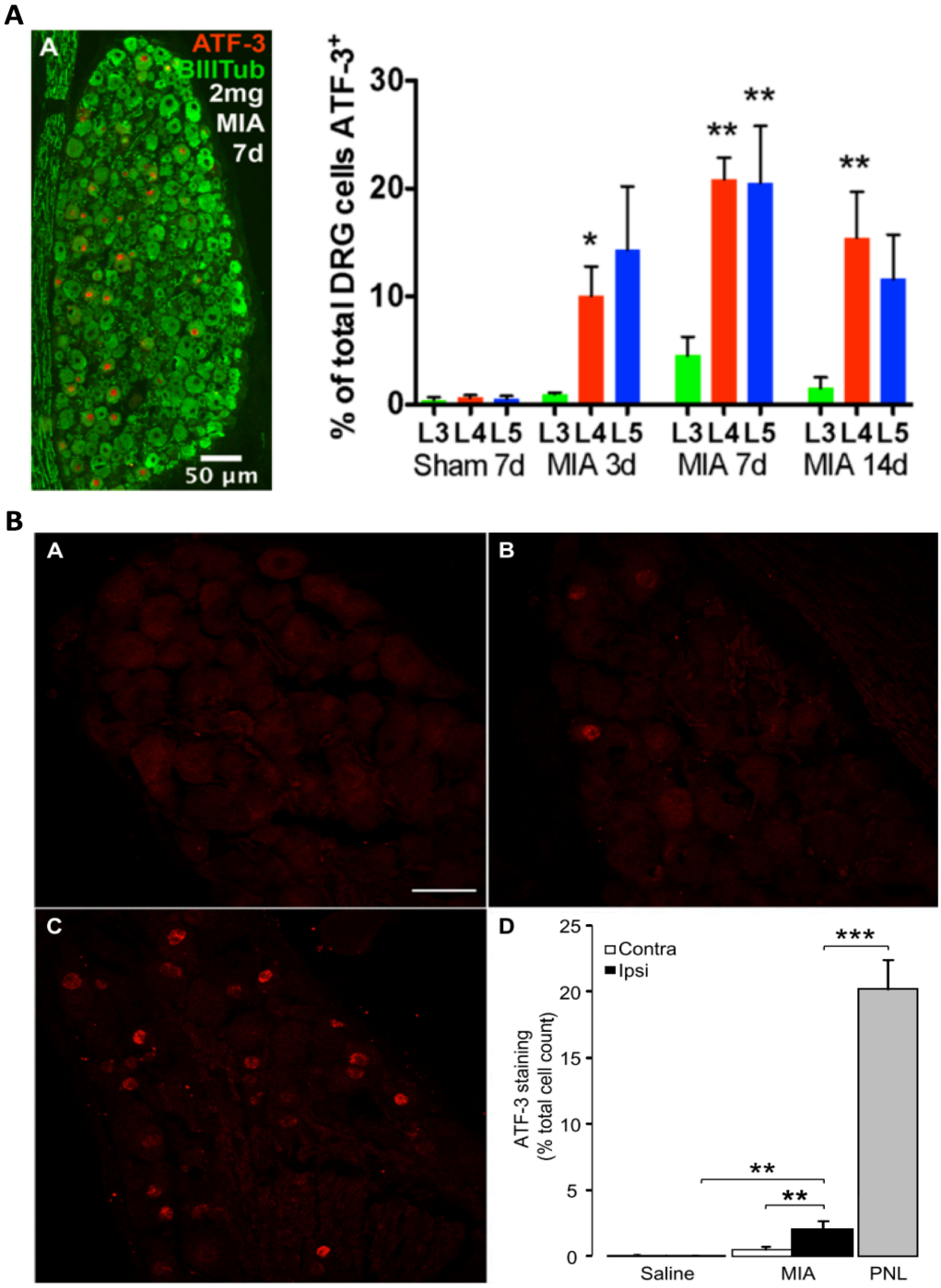


Figure 6.17 The ATF-3 expression in ipsilateral lumbar DRGs following 2mg MIA injection in the rat and 1mg MIA in the mouse. A) This figure indicates that in the 2mg MIA rat model, ATF-3 positive neurons are observed in ipsilateral L4 and L5 DRGs as early as 3 days following a 2mg MIA injection. By day 7, 20% of L4 and L5 cells bodies show positive ATF-3 staining. By day 14, the number of ATF-3 positive cell bodies begins to decline but there is still significant expression in ipsilateral L4 DRGs (Taken from Thakur et al 2012). B) This figure indicates in the 1mg MIA mouse model, although there is a significant increase in ATF-3 positive cell bodies in L3-L5 DRGs 7 days following MIA injection, this is at much lower percentages than that observed in partial nerve ligation (Taken from Ogbonna et al 2013).

Remarkably, the qPCR experiment carried out in this study indicated a significant increase in ATF-3 mRNA expression in the lumbar dorsal horn of late phase MIA animals compared to sham controls. Initially, this result was confusing as in the immunohistochemical study no ATF-3 expression was observed in the dorsal horn. However, a recently developed RNA sequencing database indicates that microglia have a large expression of ATF-3 mRNA, although immunohistochemistry suggests the mRNA is not translated to the ATF-3 protein (Figure 6.18)(Zhang et al 2014). This thesis reported a rise in Iba-1 mRNA in the dorsal horn, while previous studies have reported significant microgliosis occurs in the lumbar dorsal horn following MIA injection (See Section 5.3.4)(Thakur et al 2012, Ogbonna et al 2013). Therefore, the significant ATF-3 expression in the dorsal horn of late phase MIA animals likely reflects an increased proliferation of microglia in response to the MIA injection.

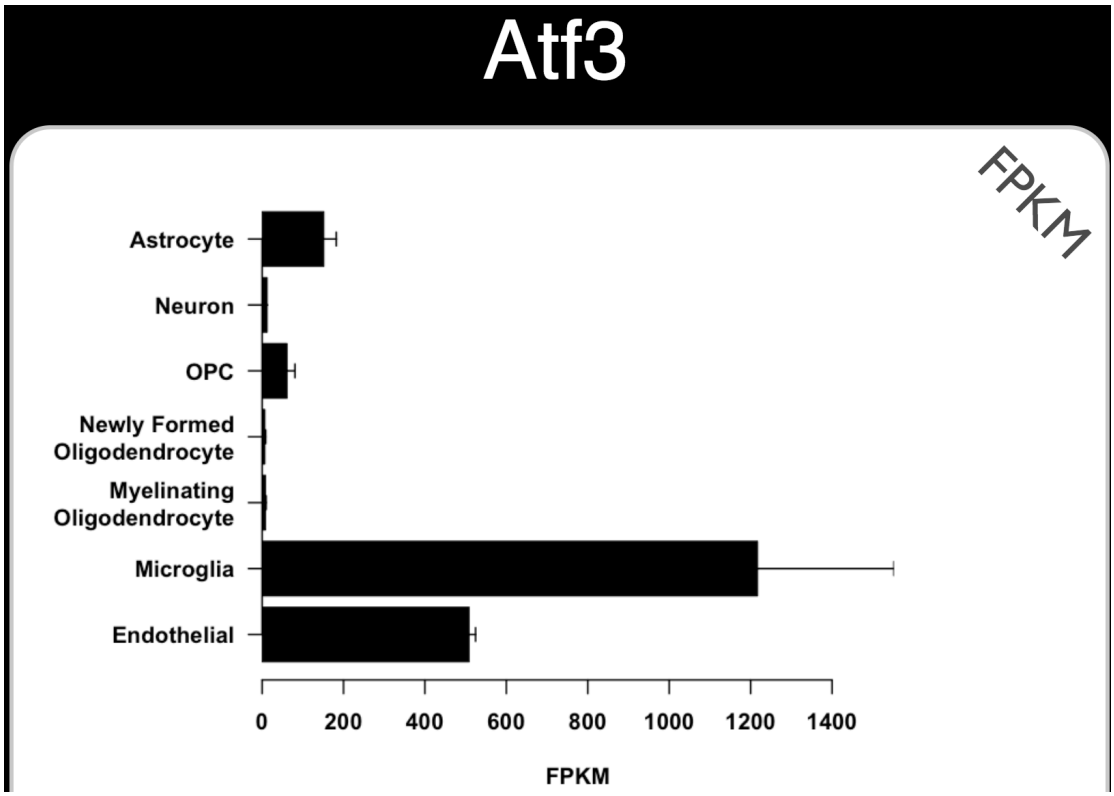


Figure 6.18 The mRNA expression of ATF-3 in various cell types. This indicates a large expression of ATF RNA (fragments per kilobase million) in microglia cells. Zhang et al 2014.

6.4.3 Concluding remarks

This study confirms that DNIC is a noradrenergic endogenous inhibitory system with a serotonergic component. This study demonstrated that enhanced noradrenergic modulation and activating spinal 5-HT_7 receptors reinstated the DNIC system in late phase MIA animals. This indicates that the loss of DNIC in late phase MIA animals is due to an imbalance in descending controls, specifically decreased descending inhibitory controls. The actions of noradrenaline or serotonin at α_2-adrenoceptors or 5-HT_7 receptors respectively, was sufficient to restore the endogenous inhibitory system, and therefore may hold promise as a therapeutic mechanism to tackle centrally driven chronic pain in OA patients.

This study also indicated that pregabalin was effective in late phase MIA animals, despite there being a lack of neuropathy. Therefore, central sensitisation and the subsequent imbalance in descending controls arising in the brainstem, which causes the abolished DNIC system, appears to present sufficient conditions for pregabalin to prove effective. Finally, a combination therapy of pregabalin and tapentadol proved effective at inhibiting pre-conditioned neuronal responses whilst also restoring the DNIC system. This indicates a combination therapy of low doses of pregabalin and tapentadol may be a feasible approach for providing effective pain relief, through synergy of their respective mechanisms.

7. General Discussion

7.1 Diffuse Noxious Inhibitory Controls

Throughout this thesis, DNIC are investigated using single unit *in vivo* recordings of convergent WDR neurons in the deep dorsal horn of the spinal cord. This thesis demonstrates that using *in vivo* electrophysiological techniques allows consistent and reliable measurement of DNIC responses, and offers the opportunity to assess the functionality of descending controls in animal models of chronic pain. Furthermore, this thesis demonstrates that even when the DNIC system appears to be abolished in animal models of chronic pain, this can be restored pharmacologically by modulating the noradrenergic and serotonergic descending systems. Therefore, pharmacologically manipulating these systems in patients may present a feasible therapeutic approach for tackling centrally driven chronic pain.

7.1.1 The importance of noradrenergic and serotonergic descending controls for the neuronal inhibition induced by DNIC

This thesis confirms that DNIC are mediated via inhibitory noradrenergic descending controls that become activated upon a noxious conditioning stimulus. Further, this thesis demonstrates that a spinal serotonergic component can influence DNIC expression, and this component may become more important following the development of central sensitisation and chronic pain.

Blocking the actions of noradrenaline at spinal α_2-adrenergic receptors with the antagonist atipamezole completely blocked the neuronal inhibition induced by a noxious conditioning stimulation in early phase MIA and sham animals that otherwise had a normally functioning DNIC system. This is in agreement with a previous study that demonstrated that atipamezole completely blocked the neuronal inhibition induced by DNIC in naïve animals (Bannister et al 2015). Together these findings indicate that under normal physiological conditions, the noxious conditioning stimulus activates descending noradrenergic controls, which carry out an inhibitory response through activating α_2-adrenergic receptors in the spinal cord.

Studies in this thesis demonstrated that DNIC expression is abolished in animal models of chronic pain, specifically in a SNL model of neuropathy and in late phase MIA animals. In addition, reduced descending noradrenergic controls acting at the α_2-adrenergic receptors in the spinal cord are demonstrated following neuropathy and in MIA animal models of chronic pain (Rahman et al 2008, Burnham and Dickenson 2013). Taken together, this suggests a downregulation of descending noradrenergic controls may be responsible for the loss of DNIC. Interestingly, blocking the inhibitory actions of noradrenaline at spinal α_2-adrenergic receptors prevented the restoration of DNIC with the SSRIs fluoxetine and citalopram in an SNL rat model of neuropathy (Bannister et al 2017). This study indicated that a tonic descending noradrenergic control is always required for DNIC to induce neuronal inhibition, even if this is at reduced levels as is the case in animal models of chronic pain.

This thesis also identified that a spinal serotonergic component can influence the expression of DNIC. In early phase MIA and sham animals that had a normally functioning DNIC system, the spinal application of the selective 5-HT_7 receptor antagonist SB-267790 reduced the level of neuronal inhibition induced by a noxious conditioning stimulation. However, unlike spinal application of atipamezole that completely abolished DNIC expression in early phase MIA animals, the spinal application of SB-267790 did not abolish DNIC, indicating that activation of spinal 5-HT_7 receptors is not crucial for DNIC expression.

An enhanced descending serotonergic facilitatory system acting at 5-HT_3 receptors in the spinal cord has been demonstrated in neuropathic and MIA animals models of chronic pain (Suzuki et al 2004, Suzuki et al 2005, Rahman et al 2009). In addition, when DNIC was abolished in a rat SNL model of neuropathy, blocking the presumed facilitatory actions of spinal 5-HT_3 receptors with the antagonist ondansetron restored neuronal inhibition induced by DNIC (Bannister et al 2015). Overall, the pharmacological studies modulating spinal 5-HT_3 receptors and 5-HT_7 receptors indicate that descending serotoninergic controls can mediate a dual mode of action on DNIC expression depending upon the spinal receptor activated. These results are in agreement with a study demonstrating that descending inhibitory and facilitatory serotonergic controls from the RVM act ultimately through the activation of spinal 5-HT_7 and 5-HT_3 receptors respectively (Dogrul et al 2009).

This thesis confirms that spinal 5-HT_7 receptors play an inhibitory role on spinal nociceptive singalling and their activation mediates the expression of DNIC. Firstly, the DNIC system was restored in an SNL model of neuropathy through enhancing the spinal synaptic content of 5-HT with the spinal administration of the SSRIs fluoxetine and citalopram, but this effect was blocked with co-administration of a selective 5-HT_7 receptor antagonist (Bannister et al 2017). This study demonstrated that increased spinal content of 5-HT produced an inhibition of spinal neuronal responses and induced DNIC expression through activating the 5-HT_7 receptor. Secondly, in the late phase MIA model the spinal application of the selective 5-HT_7 receptor agonist AS-19 restored the expression of DNIC to similar levels observed in control animals, further confirming that spinal 5-HT_7 receptors can mediate DNIC expression in states of chronic pain.

While the exact mechanism by which the spinal 5-HT_7 receptors mediated antinociception were not determined, previous findings provide potential mechanisms. Firstly, spinal 5-HT_7 receptors have been demonstrated to mediate morphine antinociception, and are ultimately responsible for mediating the antinociceptive actions of opiodergic controls arising in the RVM (Dogrul and Seyrek 2006, Dogrul et al 2009). As the MOR antagonist naloxone has been demonstrated to partially attenuate neuronal inhibition induced by DNIC, endogenous opiates are thought to contribute to the functional DNIC system, therefore spinal 5-HT_7 receptors may contribute to the neuronal inhibition induced by DNIC through opiodergic mechanisms (Le Bars et al 1981a, Kraus et al 1981). Secondly, spinal 5-HT_7 receptors are colocalised with GABAergic interneurons in the dorsal horn, and therefore the activation of 5-HT_7 receptors may mediate inhibition through the activation of inhibitory interneurons (Figure 7.1)(Brenchat et al 2010). In fact, in a sciatic nerve constriction injury model of neuropathy, an intrathecal injection of the GABA receptor antagonist bicuculline but not the MOR antagonist naloxone blocked the antihyperalgesic effects mediated by 5-HT_7 receptor activation (Viguier et al 2012). In agreement, central sensitisation is associated with disinhibtion, whereby spinal inhibitory interneurons are suppressed allowing excitatory transmission to be sustained, therefore activating spinal 5-HT_7 receptors and subsequently activating inhibitory interneurons may counteract this phenomenon (Todd 2010). Thirdly, in the sciatic nerve constriction injury model of neuropathy a decrease in neuropathy-induced over expression of IL-1β in the ipsilateral lumbar DRGs and dorsal horn was associated with the activation of spinal 5-HT_7 receptors (Viguier et al 2012). As the overexpression of cytokines due to injury may be associated with the activation of spinal glia, this may suggest that the activation of spinal 5-HT_7 receptors

influences inflammatory responses, the activation of spinal glia and the subsequent neuronal modulation. Overall, these findings could indicate that the antinociceptove actions of spinal 5-HT_7 receptors shifts from an opiodergic mechanism in the naïve state to a GABAergic mechanism in states of chronic pain.

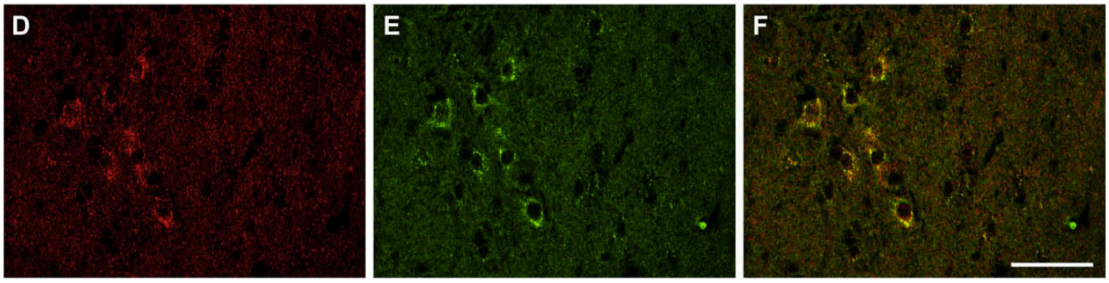


Figure 7.1. The expression of 5-HT_7 and GABAergic receptors in the dorsal horn. D) The expression of the 5-HT_7 receptor in the dorsal horn. E) The expression of GABA in the dorsal horn. F) double 5-HT_7/GABA immuno-staining in the dorsal horn indicates that receptors are co-localised. Scale bar = 50μM. (Taken from Brenchat et al 2010).

7.1.2 The applicability of testing CPM in the clinic

One important goal when studying chronic pain in animal models is to translate findings in the clinic in order that we may increase our understanding of mechanisms involved in particular pain pathways and thus recognize novel potential therapeutics. DNIC has a human counterpart, known as CPM, hence DNIC studies obtained in the lab may hold great clinical relevance if they can be translated to patients. Measuring CPM responses provides a useful tool for assessing the functionality of patients descending controls and endogenous inhibitory systems, and can subsequently provide valuable insights on treatment options for particular chronic pain states (Edwards et al 2003).

Testing the efficacy of a patients CPM system can provide insights into their physiology and likelihood of benefiting from a particular treatment. In a cohort of patients with painful diabetic neuropathy, baseline CPM was correlated with duloxetine efficacy, such that duloxetine was most effective in patients with a less efficient CPM (Yarnitsky et al 2012). In addition, a study investigating the development of post-operative chronic pain found that patients with a less efficient CPM before surgery were more susceptible to developing chronic pain after surgery (Yarnitsky et al 2008) Taken together, these studies indicate that carrying out extensive diagnosis to begin with and testing the functionality of a patients CPM system could improve future therapeutics in the long-term, through predicting how likely a patient is to develop chronic pain and which analgesics a patient may respond best to.

Importantly, the findings presented in this thesis and previous DNIC and CPM studies suggest that DNIC and CPM share a similar pharmacology. Firstly, the study demonstrating that patients with a weak CPM benefit from the SNRI duloxetine suggests that similarly to DNIC, noradrenergic and serotonergic signaling pathways subserve CPM (Yarnitsky et al 2012). In addition, the magnitude of CPM in healthy subjects has been demonstrated to be associated with serotonin transporter gene polymorphisms, indicating that similarly to DNIC in animals, CPM can be influenced by the serotonergic modulatory system (Lidstedt et al 2011). Therefore, as the CPM system in humans also relies on monoaminergic signaling, through pharmacologically modulating these systems the endogenous inhibitory system that CPM utilizes can be restored to provide effective pain relief.

Interestingly, drugs traditionally designed as antidepressants that function by modulating the levels of extracellular noradrenaline and serotonin are often effective at providing pain relief in patients with neuropathy and other chronic pain states (Bomholt et al 2005, Sindrup et al 2005). This thesis has provided further indication that DNIC and CPM are influenced by noradrenergic descending inhibitory systems, and can be modulated by a spinal serotonergic component. Due to the importance of the inhibitory descending noradrenergic system in mediating DNIC expression, this is a good first analgesic target to be considered in patients with a dysfunctional CPM system, such as NRIs that prevent the re-uptake of pre-synaptic noradrenaline such that increased levels remain in the synaptic space to carry out inhibitory actions through activating spinal α_2-adrenergic receptors (Bannister and Dickenson 2017). The finding in this thesis that Tapentadol, which functions partly as an NRI, restores DNIC in late phase MIA animals supports this approach. As serotonergic signaling can mediate both inhibitory or facilitatory influences depending upon which receptors become activated in the spinal cord, the consequences of increasing the synaptic availability of serotonin with SSRIs are more complicated. Interestingly, studies presented in this thesis demonstrate that the spinally applied SSRIs, fluoxetine and citalopram, produced antinociception in a model of neuropathy, while systemic SSRIs had no effect (Bannister et al 2017). Therefore, SSRIs could provide a second-line therapy for restoring endogenous inhibition in patients, but a more accurate therapeutic approach may be to specifically target the inhibitory serotonergic receptors, such as the 5-HT_7 receptor.

Table 7.1 summarizes the key findings on the pharmacological basis of DNIC as covered by the studies in this thesis. Overall, the similarities in the DNIC and CPM systems

indicate that DNIC studies provide promising potential for forward translation from preclinical studies to patients.

Table 7.1 The pharmacological basis of DNIC

Chronic pain model	Functional DNIC expression	Spinal receptors	
		α_2-adrenergic receptors	**5-HT$_7$ receptors**
Spinal Nerve Ligation	**x**	A descending noradrerngic tone is always required for the expression of DNIC, even when spinal 5-HT content is enhanced with SSRIs in SNL animals.	SSRIs restore DNIC in SNL animals, yet this effect is blocked by the 5-HT$_7$ receptor antagonist SB-267790.
Early Phase MIA	**✓**	An α_2-adrenergic receptor antagonist completely blocks DNIC expression in early phase MIA animals.	A 5-HT$_7$ receptor antagonist SB-267790 reduces DNIC expression but does not block it in early phase MIA animals.
Late Phase MIA	**x**	The NRI Tapentadol restores DNIC expression in late phase MIA animals.	A 5-HT$_7$ receptor agonist AS-19 restores DNIC in late phase MIA animals.
Previous studies	**N/A**	1. The α_2-adrenergic receptor antagonists atipamezole and yohimbine block DNIC expression in naïve animals. 2. The NRIs Reboxetine and Tapentadol restored DNIC expression in an SNL model of neuropathy. 3. Descending inhibitory noradrenergic controls are downregulated in animal models of neuropathic and MIA induced chronic pain.	1. Spinal 5-HT$_7$ receptors play an antinocicptive role in neuropathic and inflammatory animal models. 2. Spinal 5-HT$_7$ receptors are colocalised with GABAergic cells –may mediate neuronal inhibition through activation of inhibitory interneurons. 3. Increased density of 5-HT$_7$ receptors following neuropathy – potential for modulation of this system in chronic pain.
References	**N/A**	1. Bannister et al 2015 2. Bannister et al 2015 3. Rahman et al 2008, Burnham and Dickenson 2013.	1. Brenchat et al 2009, Brenchat et al 2010 2. Viguier et al 2012 3. Brenchat et al 2010

7.2 The monoiodoacetate model of Osteoarthritis: translational relevance to the clinic

A further objective of this thesis was to better understand the chronic pain associated with OA, using a 2mg MIA rat model. Chronic pain associated with OA is a heterogeneous condition, with patients presenting with several and varied symptoms, which become increasingly complicated and difficult to interpret due to the discordance between radiological joint damage and the associated pain phenotype (Hannan et al 2000, Malfait and Schnitzer 2014). Studies in this thesis investigated the MIA induced joint damage and the associated pain-like behaviour. This thesis specifically focused on exploring MIA-induced central sensitisation and the associated dysregulation of descending controls arising in the brainstem, which was assessed through measuring the DNIC responses in early and late phase MIA animals. In addition, this thesis examined the innervation of the knee and the potential for an MIA-induced neuropathic component contributing to the associated chronic pain. Importantly, the findings presented in this thesis demonstrate subtypes of the MIA model, which may correspond to patient segments who may respond best to particular therapeutic interventions.

7.2.1 Knee histology and associated pain-like behaviour

While it is difficult to exactly mimic the aetiology of osteoarthritis and associated chronic pain, an MIA injection in the intrarticular space aims to cause similar joint damage to that observed in patients. Therefore, the level of cartilage damage caused by the MIA injection and the associated chronic pain was assessed in early and late phase MIA animals.

Interestingly, the knees taken from early phase MIA animals showed no significant cartilage damage, despite the development of significant pain like behaviour in these animals. Although findings in this thesis demonstrate no increased mRNA expression levels of the pro-inflammatory cytokines IL-1β, IL-6, and TNFα, in the ipsilateral lumbar DRGs, the levels of these pro-inflammatory cytokines were not investigated in the joint, which is where an inflammatory infiltrate has been reported in both MIA animals and OA patients. Therefore, this study did not rule out the possibility of an inflammatory infiltrate within the joint, which could be driving peripheral sensitisation. MIA model studies have demonstrated a significant inflammatory infiltrate occurs in the joint

rapidly after injection (Guzman et al 2003, Clements et al 2009, Orita et al 2011). There is significant increase in the levels of white blood cells and pro-inflammatory cytokines in the joint in the first week following MIA injection, with levels peaking around day 3-4 (Guzman et al 2003, Clements et al 2009, Orita et al 2011). In addition, although OA is not traditionally considered an inflammatory condition, an inflamed synovium and the subsequent release of pro-inflammatory cytokines are linked with cartilage degradation in OA patients, indicating joint inflammation is associated with an accelerated disease pathogenesis (Sellam and Berenbaum 2010). Overall, the early phase MIA model may present a useful tool for exploring the inflammatory component associated with OA but may not accurately represent cartilage joint damage observed in most OA patients.

On the other hand, in knees taken from late phase MIA animals there was significant cartilage damage. This is in agreement with other MIA-induced knee pathology studies, which identified severe cartilage damage and proteoglycan loss, combined with initial damage to the subchondral bone, such as sclerosis, lesions and fragmentation (Guingamp et al 1997, Guzman et al 2003). Interestingly, a study in this thesis found a positive correlation between the extent of cartilage damage and the associated pain-like behaviour, such that animals with more extensive cartilage damage avoided placing weight on the injured joint and showed increased levels of secondary hyperalgesia on the hind paw. This indicates that the pain like behaviour that develops in the late phase MIA model is an accurate representation of joint damage associated chronic pain, and provides a valid translational tool to OA patients.

The histological findings in this thesis indicate how important the time frame is for the extent of MIA-induced joint damage, and that a lengthier model may more accurately represent the OA joint damage observed in patients. As discussed, early phase MIA animals could provide a used tool for further exploration of the inflammatory component to OA. Meanwhile, late phase MIA animals demonstrated extensive cartilage damage and translational studies may help to further our understanding of cartilage and joint damage induced development of chronic pain. However, a lengthier MIA model may allow for assessment of chronic pain associated with subchondral bone damage and remodeling, which can be observed in severe cases of OA in patients (Buckland-Wight 2004, Brandt et al 2006).

7.2.2 The expression of DNIC and associated central changes in the MIA model

The main focus of this thesis involved assessing the expression of DNIC in the MIA model, which proved a useful tool for investigating the functional state of descending controls.

This thesis demonstrated that early phase MIA animals have a functional DNIC expression, with neuronal inhibition induced by a noxious conditioning stimulus at levels similar to those observed in naïve and sham control animals. As it has been indicated in a cohort of patients with diabetic neuropathy that those with a functional CPM do not benefit as well from the SNRI duloxetine, it may indicate that OA patients with a functional CPM may also not necessarily benefit from therapies that target monoaminergic signaling and aim to restore this system (Yanritsky et al 2012). In addition, previous studies have demonstrated that rats that received low doses of MIA (50mg/ml) and had limited joint damage were sensitive to NSAIDs, while rats that received high doses of MIA (80mg/ml) develop NSAID resistant ongoing pain (Havelin et al 2016). Furthermore, in a 2mg MIA model the NSAID Diclofenac only proved effective 3 days after MIA induction, which coincided with knee swelling (Fernihough et al 2004). As an extensive inflammatory infiltrate has been demonstrated in early phase MIA animals, it would indicate that tackling the inflammatory component to the associated pain with NSAIDs would be a feasible approach (Guzman et al 2003, Fernihough et al 2004, Clements et al 2009, Orita et al 2011). These findings could be translated and explored further in the clinic, such that OA patients who present with joint inflammation and an efficient CPM system may benefit most from NSAIDs that act to tackle the peripheral inflammatory source of pain rather than therapeutic interventions that aim to tackle centrally driven chronic pain.

On the other hand, late phase MIA animals had abolished DNIC expression, as there was no inhibition of mechanically evoked neuronal firing with a concurrent noxious ear pinch. The DNIC system was restored with the NRI and MOR agonist Tapentadol, and by activating spinal 5-HT_7 receptors, indicating that the system was not completely abolished but rather masked by an imbalance in descending controls. Previous studies have indicated that as the MIA model progresses, NSAID resistant ongoing pain develops (Fernihough et al 2004, Havlin et al 2006) Furthermore, patients have been demonstrated to develop referred pain and hypersensitivity, and a subset of patients remain with chronic pain following total joint replacement surgery (Gwilym et al 2009,

Wylde et al 2011). These findings indicate that late phase MIA animals and a subset of OA patients develop pain that can no longer be the result of purely peripheral mechanisms. Therefore, therapeutic interventions that aim to tackle the centrally driven contribution to OA associated chronic pain may be most appropriate in this subset of patients (See Section 7.1.2). Specifically, if OA patients present with a dysfunctional CPM system, the findings presented in this thesis suggest treatments with the most benefit would act to restore CPM expression through pharmacologically modulating the balance in descending inhibitory and facilitatory controls.

Investigating the expression of DNIC in the MIA model of OA has provided information on how central changes and an imbalance in inhibitory and facilitatory descending controls do not appear to develop until the later stages of disease progression. This can be applied to the OA clinic, as through assessing both the level of joint inflammation and the efficiency of CPM, valuable insights can be gained on the most effective analgesic approach for individual patients.

7.2.3 A neuropathic component to the MIA model

A study presented in this thesis indicated that while Pregabalin proved ineffective at producing an inhibitory effect in early phase MIA animals, Pregabalin had a significant inhibitory effect on neuronal firing in late phase MIA animals. As gabapentinoid drugs have proved effective treatments in neuropathic conditions, this may have indicated a neuropathic component to the MIA-induced pain (Thakur et al 2014, Patel and Dickenson 2016). However, ATF-3 staining in the ipsilateral lumbar DRGs suggested there was no MIA induced damage to peripheral afferents innervating and surrounding the knee. In addition, there was no upregulated mRNA expression of the $\alpha_2\delta_1$ subunit of VGCCs in the ipsilateral lumbar DRGs or dorsal horn, which has been reported in animal models of neuropathy (Bauer et al 2009). The lack of ATF-3 staining and no change in the mRNA expression of the $\alpha_2\delta_1$ subunit conflict with previous MIA studies (Rahman et al 2009, Thakur et al 2012). As the same dose of MIA (2mg) in the same amount of saline (25μL) was used for these studies, the discrepancies in results could come down to injection technique and differing levels of neuropathy between the models.

The results presented in this thesis suggest that there was no neuropathic component to the MIA induced pain and that late phase MIA animals were sensitive to Pregabalin due to other mechanisms, such as upregulated facilitatory serotonergic descending controls

acting at 5-HT_3 receptors (Suzuki et al 2005). Nevertheless, the Fast Blue neuronal tracer study did demonstrate that the knee joint is innervated, and so OA induced joint damage does hold the potential for neuropathy. Furthermore, if the MIA model had been left to progress for longer this may have led to subchondral bone fibrillation and lesions, and as the subchondral bone contains many free nerve endings, this would likely result in peripheral nerve damage and may reflect the more advanced cases of OA observed in patients (Guingamp et al 1997, Guzman et al 2003, Aso et al 2014). Overall, although a neuropathic component to the MIA model was not identified in this thesis, a subset of patients still present with neuropathic features and this should be considered when deciding on the appropriate therapeutic intervention (Thakur et al 2014).

7.2.4 Personalised medicine in the OA clinic

Importantly, the findings presented indicate that accurate diagnoses and personalised medicine approaches could be explored further in the clinic and may be the most promising approach for providing effect pain relief in patients with OA. As discussed, accurately testing the levels of inflammation, efficiency of CPM, and development of neuropathy, would provide valuable insights into which therapeutic intervention would likely produce the most effective pain relief. In addition, a combination therapy of Pregabalin and Tapentadol was found to efficiently inhibit pre-conditioned neuronal firing, and also reinstate DNIC. This suggests that combination therapies may be a useful approach for targeting different mechanisms of OA induced chronic pain. For instance, if an OA patient presented with neuropathic features and an inefficient CPM, this treatment approach could tackle both components. Not only will accurate diagnostics and a personalised medicine approach provide a better quality of care to patients, it will also avoid wasteful expenditure on treatments that would prove ineffective in a subset of patients.

Table 7.2 summarizes the key findings on the aeitiology of the MIA model as covered by the studies in this thesis compared to previous studies investigating the MIA model.

Table 7.2 The aeitiology of the MIA model

Feature	Stages of MIA model			
	Early phase MIA	**Late phase MIA**	**Other studies**	**References**
Joint Histopathology	Little articular cartilage degradation.	Significant articular cartilage degradation and exposure of subchondral bone.	14 days post-injection: Severe cartilage damage and proteoglycan loss. Initial damage to subchondral bone, including sclerosis, fragmentation, bone lesions.	Guingamp et al 1997 Guzman et al 2003 Mohan et al 2011
Peripheral Inflammation	No increase in mRNA expression of pro-inflammatory cytokines in ipsilateral lumbar DRGs.	No increase in mRNA expression of pro-inflammatory cytokines in ipsilateral lumbar DRGs.	Significant inflammatory infiltrate in joint soft tissue structures in early phase MIA animals, which declines over time.	Guzman et al 2003 Clements et al 2009 Orita et al 2011
Peripheral nerve damage	No ATF-3 positive staining in ipsilateral lumbar DRGs.	No ATF-3 positive staining in ipsilateral lumbar DRGs.	A significant increase in the number of ATF-3 positive cell bodies in L4 and L5 DRGs, 3, 7 and 14 days post MIA injection. A significant increase in ATF-3 positive cell bodies in L4 DRG 14 days post injection. ATF-3 staining observed in 2.1% of L3, L4 and L5 DRG cells 7 days post injection.	Thakur et al 2012 Orita et al 2011 Ogbonna et al 2013
Sensitivity of dorsal horn neurons	No increase in mechanically evoked WDR	No increase in mechanically evoked WDR	Enhanced WDR neuronal firing rates in response to	Rahman et al 2009 Burnham and

	neuronal firing rates compared to sham controls.	neuronal firing rates compared to sham controls.	noxious stimulations. Enhanced spontaneous firing rate of WDR neurons.	Dickenson 2013 McGaraughty et al 2010 Chu et al 2011
Microgliosis	No significant increase in the mRNA expression of Iba1 or pro-inflammatory cytokines in the dorsal horn.	A rise in the mRNA expression of Iba1 and the pro-inflammatory cytokine IL-1β, but this did not reach significance.	Significant shift of microglia into effector morphometry by day 7. Significant proliferation of microglia in the ipsilaterl lumbar spinal cord.	Thakur et al 2012 Sagar et al 2011 Ogbonna et al 2013
Functionality of descending controls	Functional DNIC expression.	Abolished DNIC expression.	Endogenous noradrenergic inhibition was reduced in late phase MIA animals. Enhanced serotonergic excitatory drive modulates evoked neuronal responses in late phase MIA animals.	Burnham and Dickenson 2013 Rahman et al 2009

7.3 Methodological considerations

While the preclinical investigation of animal models of chronic pain expands our understanding of the neurobiology of pain and provides useful insights into the molecular mechanisms of effective analgesics, any differentiations between animal and human pathways needs to be considered. An awareness of the limitations of each experimental technique will allow for the most accurate conclusions to be drawn, and for appropriate translation from preclinical models to the clinic.

7.3.1 Single-unit *in vivo* recordings

7.3.1.1 The use of supra threshold stimulations

Single unit *in vivo* recordings provide a useful technique for unbiased measurements of neuronal responses to natural and electrical stimulations, and in turn an understanding of how these responses can be modulated by pharmacological manipulation in animal models of chronic pain. Many other experimental techniques used in animals models of preclinical pain research, such as behaviour and electromyographic recordings, measure nociceptive responses based on reflex withdrawals, yet it remains questionable how reliably reflex withdrawals reflect the human pain experience (Mogil 2009). In addition, behavioural techniques only allowing for threshold level stimulations due to ethical reasons, there is a also a subjective nature to the tests, which may introduce bias. In contrast, single-unit *in vivo* recordings allows for measurements of neuronal responses to supra threshold stimuli, which are likely to reflect the high pain levels reported by patients in the clinic (Sikander and Dickenson 2013).

7.3.1.2 The search strategy for neurones

There are limitations to single unit *in vivo* recordings that should be taken into consideration. Firstly, the strategy employed for finding neurons ensures a sample of neurons are selected with similar characteristics, and whilst this allows for comparisons between studies it may also introduce bias. For example, neurones with a high level of baseline spontaneous activity tend to be excluded from studies as they are highly excitable and are likely to burst following a few noxious stimulations to the receptive field. In addition, a high level of tonic spontaneous activity makes it difficult to distinguish between spontaneous and evoked action potentials, and therefore makes DNIC induced neuronal inhibition difficult to interpret. However, WDR neurons receiving input from both the knee and hind paw in MIA animals have been demonstrated to have an enhanced spontaneous firing rate compared to Shams (McGaraughty et al 2010, Chu et al 2011). Therefore, excluding neurons with high levels of spontaneous firing may produce an underestimation of central sensitisation in MIA animals. This may provide one explanation for why mechanically evoked neuronal responses were not found to be enhanced compared to shams in this thesis.

7.3.1.3 Descending controls and anaesthesia

It has previously been considered that for CPM, the conditioning stimulus may attenuate the pain response partly through a distraction mechanism. However, human studies have confirmed that the CPM system acts independently from distraction (Moont et al 2010). The results presented in this thesis with anaesthetized animals provide unequivocal evidence that the noxious conditioning stimulus induces neuronal inhibition through a mechanism that does not involve distraction. As the low level of anaesthesia used is constant for the duration of the experiment, and the same levels are used between animals, any anaesthesia-induced reduction in spinal neuronal activity should also remain constant and allows for comparisons between studies. However, when using recordings from anaesthetized animals, the effect anesthesia may have on descending modulation needs to be considered, especially when taking into account that isoflurane has been reported to mediate antinociception through noradrenergic descending controls acting at spinal α_2-adrenergic receptors (Kingery et al 2002). If isoflourane were modulating descending controls through this mechanism, outcomes may have to be interpreted more carefully. However, the alterations observed in the descending noradrenergic system in late phase MIA and SNL animals compared to shams, when all animals are under the same level of isoflurane indicates that isoflurane is not modulating descending noradrenergic activity enough to effect DNIC repsonses. Furthermore, the potent effects produced by the α_2-adrenergic receptor antagonist atipamezole and the NRIs Reboxetine and Tapentadol suggests there is little competition for spinal α_2-adrenergic receptors, further indicating isoflurane is not be having a large effect on these receptors.

7.3.1.4 The DNIC protocol

In the MIA model, DNIC was induced with both a concurrent noxious ear pinch and knee pinch. The concurrent noxious pinch was placed on the MIA injured knee to assess if noxiously stimulating the area of primary hyperalgesia could induce neuronal inhibition. However, in both animal DNIC studies and human CPM paradigms the conditioning stimulus is placed distant to the test stimulus (Bannister and Dickenson 2016a, Yarnitsky 2015). Therefore, as the test stimulus is placed on the receptive field of the hind paw, and the concurrent conditioning stimulus is placed on the ipsilateral MIA injured knee, these two sites may be too close to constitute a DNIC response. As such, the inhibition observed in neuronal firing upon concurrent noxious MIA injured knee pinch in late phase MIA animals, who otherwise demonstrate an abolished DNIC system, may be due to other mechanisms rather than DNIC. However, DNIC is abolished in both

ipsilateral and contralateral WDR neurons in late phase MIA animals. Therefore, the DNIC expression was also assessed in contralateral cells with a concurrent noxious pinch placed on the MIA injured knee, which is now contralateral to the hind paw receptive field and would count as a distant site.

7.3.2 The MIA model

As discussed in Section 5.1.1, although it is difficult to mimic the exact aetiology of OA in an animal model, the 2mg MIA model produces similar joint pathology, associated pain-behaviour and evidence of central sensitisation. Although there is an initial inflammatory infiltrate into the joint rapidly following MIA injection, the inflammatory response begins to subside by day 7 and continues to stay low at the later stages of the model (Guzman et al 2003, Orita et al 2011). In contrast, although a higher inflammatory infiltrate was found in joint tissue from patients with early phase OA, patients present with episodic synovial inflammation, which occurs in both the early and late stages of the disease (Benito et al 2005, Scanzello and Goldring 2012). Therefore, although the inflammatory response occurring in the early stages of the MIA model represents similarities with the human OA condition, the fact that late phase MIA models develop NSAID insensitive ongoing pain may not necessarily be reflective of patients (Fernihough et al 2004, Havelin et al 2016). While NSAIDs may prove generally ineffective in patients with advanced OA, they may prove beneficial in OA patients undergoing a synovitis flare, which can be detected through MRI or ultrasound imaging of the joint (Scanzello and Goldring 2012). Overall, while the late phase MIA model shares similar structural joint changes with OA patients, it may not characterize inflammatory flares observed in OA patients, and therefore may not be a perfect model for forward translation to the clinic in every case.

7.3.3 MIA induced histopathology of the knee

While cartilage damage has long been considered the central feature for initiating OA, and MIA was shown to significantly damage articular cartilage in late phase animals, it is becoming increasingly clear that OA should be considered a disease of the whole joint (Loeser 2006, Dieppe 2011). Although the knee histology presented in this thesis showed significant degradation of the articular cartilage and exposure of subchondral bone in late phase MIA animals, it did not assess any damage to the synovium, subchondral bone or surrounding joint structures, such as the muscles and ligaments. Although the specific OA induced joint damage varies greatly between patients, damage can occur in all subchondral joint structures and contribute to the development of

disease pathogenesis (Wieland et al 2005)(See Section 1.3.2). As this thesis explores the chronic pain associated with OA, and the cartilage itself is not innervated and so cannot be causing pain directly, it may be beneficial to use a method that allows for assessing damage to the whole joint. Furthermore, one feature of OA induced joint damage associated with flares of chronic pain in patients is bone marrow lesions (Felson et al 2001, Zhang et al 2011). Therefore, a technique such as microCT, which is capable of detecting subchondral joint damage, may allow for a clearer understanding of what may be driving the associated chronic pain and central sensitisation in the MIA model (Morenko et al 2004). Overall, assessing the damage caused by MIA to the joint as a whole may provide valuable insights on how translatable the MIA induced joint damage is compared to OA induced joint damage in patients.

7.3.4 The limitations of qPCR

In this thesis, changes in the mRNA expression of monoaminergic receptors, glia biomarkers, pro-inflammatory cytokines, and nerve injury markers were assessed in the ipsilateral lumbar DRGs and dorsal horn using qPCR. While little changes in the mRNA expressions were reported in this thesis, it should be considered that this might due to a dilution effect. Firstly, the whole of the lumbar dorsal horn was taken and used for RNA extraction, while changes in mRNA expression may occur in a specific areas of the dorsal horn. Secondly, the cellular heterogeneity of the DRGs and dorsal horn should be taken into account. When investigating monoaminergic receptors and nerve injury markers, the interest was specifically based on changes to mRNA expression in the neurons. However, in the dorsal horn the glia cells alone outnumber neurons ten to one (McMahon and Koltzberg 2013). Similarly, it has been demonstrated that from a dissociated preparation of whole ganglia, 10% of the composition constitutes a mixture of neuronal cells, while the remaining 90% of this preparation is made up of satellite ganglia and other non-neuronal cell types (Thakur et al 2014b). When it is considered that only certain neurons will express the receptor of interest, this dilutes the chances of seeing an effect further still. One method that could be employed to remove the diluting effect would be to separate the neurons from the rest of the tissue. A magnetic purification technique has been demonstrated to achieve this in DRG tissues, while a single nucleus RNA-sequencing technique can provide identification of neuronal populations in the spinal cord (Thakur et al 2014b, Sathyamurthy et al 2018). Overall, it should be considered that the lack of significant differences observed with qPCR in this thesis does not necessarily mean there were no changes to receptor expression in the

neurons, it may just be that the effect was too small to be detected in whole tissue preparations.

7.4 Future studies

This thesis demonstrated that testing the functionality of DNIC using in vivo single unit recordings of convergent WDR neurons provides a robust and reliable technique for assessing the function of descending controls in animal models of chronic pain. This thesis further demonstrates that in the late phase MIA model of OA, where there is substantial cartilage damage, the DNIC system is abolished due to an imbalance in descending inhibitory and facilitatory controls. In addition to the amendments to the experimental techniques discussed above, further studies could be carried out to better understand the exact molecular mechanisms underlying the joint damage induced barrage of nociceptive information and the subsequent imbalance in descending controls and abolished DNIC expression.

7.4.1 The contribution of joint damaged induced peripheral nociceptive drive and the dysfunctional DNIC system

This thesis demonstrated that significant cartilage damage was not observed until the late phase of the MIA model, and that a longer time course of the model may provide a tool for further exploration of subchondral bone damage induced central changes. Interestingly, DNIC expression is abolished as the model progresses to 14 days. It has previously been demonstrated that the DNIC system is lost in a SNL rat model of neuropathy (Bannister et al 2015, Bannister et al 2017). However, and as the articular cartilage is not innervated and no other signs of neuropathy were detected in the MIA model in this thesis, it suggests that the modulation of descending inhibitory and facilitatory controls and the subsequent abolished DNIC expression can occur in other pain models, even without neuropathy.

A substantial inflammatory infiltrate has been demonstrated in early phase MIA animals and in OA patients, and it should be considered that this could be responsible for the development of peripheral sensitisation (Guzman et al 2003, Clements et al 2009, Scanzello and Goldring 2012). Furthermore, the barrage of nociceptive signals from the periphery and the subsequent continuous stimulation of second order neurons may result in sensitisation of dorsal horn neurons (Woolf 2011). Therefore, it would be interesting to assess how inhibiting this peripheral nociceptive drive early in the MIA

model impacts the development of central sensitisation and the subsequent imbalance in descending controls.

Firstly, NSAIDs are effective at providing pain relief in early phase MIA animals (Fernihough et al 2004, Havelin et al 2016). Similarly, NSAIDs provide effective analgesia in the early stages of OA in patients, through preventing the synthesis of prostaglandins and preventing peripheral sensitisation that arises due to the inflammatory component of OA (Lin et al 2004, Wieland et al 2005). Secondly, the neurotrophin NGF is part of the 'inflammatory soup' produced in response to tissue damage and is thought to contribute to peripheral sensitisation as it sensitizes TRPV1 channels on peptidergic C fibres, especially in inflammatory conditions such as OA (McMahon 1996). In addition, the NGF neutralizing antibody Tanezumab has been demonstrated to reduce joint pain in patients with moderate to severe OA of the knee joint (Lane et al 2010). An interesting approach for assessing the impact of peripheral sensitisation could be to induce MIA damage and then apply topical NSAIDs or NGF neutralizing antibodies to the knee and assess if the DNIC system still becomes dysfunctional. This could provide valuable insights for treatment options in the clinic and address the option of targeting the peripheral component early on disease pathogenesis with the aim to prevent the development of centrally driven chronic pain.

7.4.2 Further assessing DNIC pharmacology at the level of the spinal cord

As discussed in Chapter 4, the descending serotonergic system exerts a bi-directional role on pain processing as both inhibitory and facilitatory actions have been demonstrated upon 5-HT release in the dorsal horn. There are a myriad of serotonergic receptors in the spinal cord, and the overall pronociceptive or antinociceptive action of descending serotonergic controls depends upon the receptors subtypes activated. The role of spinal 5-HT_3 receptors in pain transmission have been extensively studied, and play a facilitatory role in pain processing in MIA animals (Rahman et al 2009). Furthemore, blocking the actions of 5-HT at spinal 5-HT_3 receptors restores DNIC in a model of neuropathy (Bannister et al 2015). This thesis investigated the tole of spinal 5-HT_7 receptors in pain transmission. The spinal co-administration of the 5-HT_7 receptor antagonist SB-267790 completely blocked the inhibitory actions of the SSRIs fluoxetine and citalopram, which indicates a principal role of this receptor in mediating serotonergic inhibition (Bannister et al 2017). These findings agree with previous studies, that spinal 5-HT_3 receptors mediate facilitation, while spinal 5-HT_7 receptors mediate and inhibition of the pain response (Dogrul and and Seyrek 2006, Dogrul et al

2009). However, it would be interesting to use antagonists and agonists for the remaining spinal serotonergic receptors in early and late phase animals to better understand the specific roles of each receptor in the expression of DNIC.

7.4.3 The brain areas involved in the development of a dysfunctional DNIC system

As the DNIC system is lost following spinal transection, it indicates that DNIC requires supraspinal signals and the activation of functional descending controls, however the exact brain signaling pathways required for the expression of DNIC are yet to be conclusively determined (Le Bars et al 1979b, Dickenson and Le Bars 1983). Changes in the level of activity in the SRD brainstem nuclei have been demonstrated to be crucial for the expression of DNIC in animals and CPM in humans (Bouhassira et al 1992a, Youssef et al 2015). However, complex interactions between the SRD, higher brain structures in the cortex, and midbrain and brainstem structures that comprise the dorsolateral funiculus are thought to be required for the activation of descending monoaminergic controls, which are ultimately responsible for mediating DNIC (See Section 1.2.4)(Youssef et al 2016, Bannister and Dickenson 2017).

As the MIA model is a robust and easily inducible animal model of chronic pain, where DNIC is functional in the early phase but abolished in the late phase, this model offers the opportunity for assessing changes in the neuronal activity of brain structures reported to be involved in the efficacy of DNIC. In human CPM studies, fMRI identified a reduction of activity within the SRD upon a concurrent noxious conditioning stimulus, which was responsible for mediating the CPM response (Youssef et al 2015). Furthermore, fMRI highlighted the involvement of the dlPFC, CC, amygdala and Pb nucleus in modulating the CPM response (Youssef et al 2016). Therefore, this technique could be back translated to the MIA model, to better understand changes in activity occurring in the brain as the model progresses and the DNIC system becomes abolished. In addition, the resolution of fMRI allows for the observation of neuronal activity across the entire brain, which may allow for a better understanding of how brain structures interact to induce a DNIC response, and highlight any alterations in these connections when DNIC is lost in late phase animals (Ferris et al 2006).

Despite the advantages of fMRI, this is an expensive technique that requires sophisticated equipment. Another technique that could be employed to further dissect the brain structures responsible for inducing DNIC is brain microinjections and blocking

or activating specific brain regions. In addition, brain microinjections could be utilised while simultaneously recording DNIC responses in WDR neurons using *in vivo* single unit recordings, which would be difficult to combine with fMRI. Therefore, a similar approach could be taken as that presented in this thesis, where the DNIC response is evaluated in convergent WDR neurons while pharmacologically manipulating the monoaminergic descending controls. However, in this scenario the descending controls would be pharmacologically manipulated at supraspinal levels rather than at the level of the spinal cord. A further approach could be to take electrophysiological recordings from the brain structures reported to be involved in the DNIC system, and assess any changes in neuronal activity between early and late phase MIA animals. Overall, in vivo electrophysiological techniques provide a reliable method for assessing DNIC in animal models of chronic pain, and hold potential for unraveling the roles and interactions played by supraspinal structures in activating descending controls and inducing neuronal inhibition at the level of the spinal cord.

7.5 Concluding remarks

Firstly, this thesis confirms that assessing DNIC responses using *in vivo* single unit recordings of convergent WDR neurons in the dorsal horn provides a robust and reliable technique for examining the functional state of descending controls in animal models of chronic pain. Importantly, as DNIC and CPM share similar pharmacology and altered efficiencies in states of chronic pain, this electrophysiological technique provides a useful tool for forward translation to the clinic (Yarnitsky 2010, Yarnitsky 2015). Overall, the work presented in this thesis confirms previous findings that DNIC represents a noradrenergic endogenous inhibitory system with a partly descending serotonergic component (Bannister et al 2017).

Secondly, the work presented in this thesis investigated an MIA induced model of OA in the rat. It was demonstrated that although both early and late phase MIA animals develop pain in both the knee joint and ipsilateral hind paw, only late phase MIA animals display significant articular cartilage damage in the knee joint. Due to the joint damage induced continuous barrage of nociceptive signaling from the periphery and the high level of neuronal plasticity in the CNS, it is likely that sensitivity also develops in second order neurons in the dorsal horn. However, the results presented in this thesis did not suggest an increase in evoked activity of WDR neurons, which contrasts with previous findings in the MIA model (Rahman et al 2009, Burnham and Dickenson 2013)

However, a rise in the mRNA levels of the pro-inflammatory cytokine IL-1β and the microglia biomarker Iba1 were detected in the dorsal horn of late phase animals suggesting the development of microgliosis, which is in agreement with previous findings in the MIA model and is indicative of central sensitisation (Thakur et al 2012, Ogbanna et al 2013).

Furthermore, DNIC is abolished in late phase MIA animals, indicative of an imbalance in descending inhibitory and facilitatory controls. As an increased serotonergic facilitatory drive has previously been demonstrated in MIA animals, and blocking spinal 5-HT_3 receptors has been demonstrated to restore DNIC in a model of neuropathy, it is likely there is an increased serotonergic facilitatory drive acting at spinal 5-HT_3 receptors contributing to the loss of DNIC in late phase MIA animals (Rahman et al 2009, Bannister et al 2015). A study in this thesis demonstrated that DNIC expression could be restored in late phase animals with the NRI Tapentadol, suggesting a decreased noradrenergic inhibitory drive also contributes to the loss of DNIC. In addition, activating spinal 5-HT_7 receptors restored DNIC expression in late phase MIA animals. As the 5-HT_7 receptors are colocalised with GABAergic inhibitory interneurons and disinhibition is reported in states of central sensitisation, it is likely there is decreased serotonergic inhibitory tone acting at spinal 5-HT_7 receptors and a subsequent decrease in the activation of inhibitory interneurons in the dorsal horn (Todd 2012, Brenchat 2010, Viguier et al 2012). A general schematic of the peripheral joint damage and centrally driven chronic pain is shown in Figure 7.2. Overall, the studies presented in this thesis indicate that restoring the balance in descending inhibitory and facilitatory controls through pharmacologically manipulating monoaminergic signaling is an effective approach for providing analgesia in the MIA model of OA.

I hope the studies presented in this thesis further our understanding of how modulation of descending controls can influence the chronic pain associated with OA. I hope through assessing CPM efficiency in the OA clinic, patient segmentation and a personalised medicine approach could be adopted such that each individual patient could receive the most appropriate analgesic therapy.

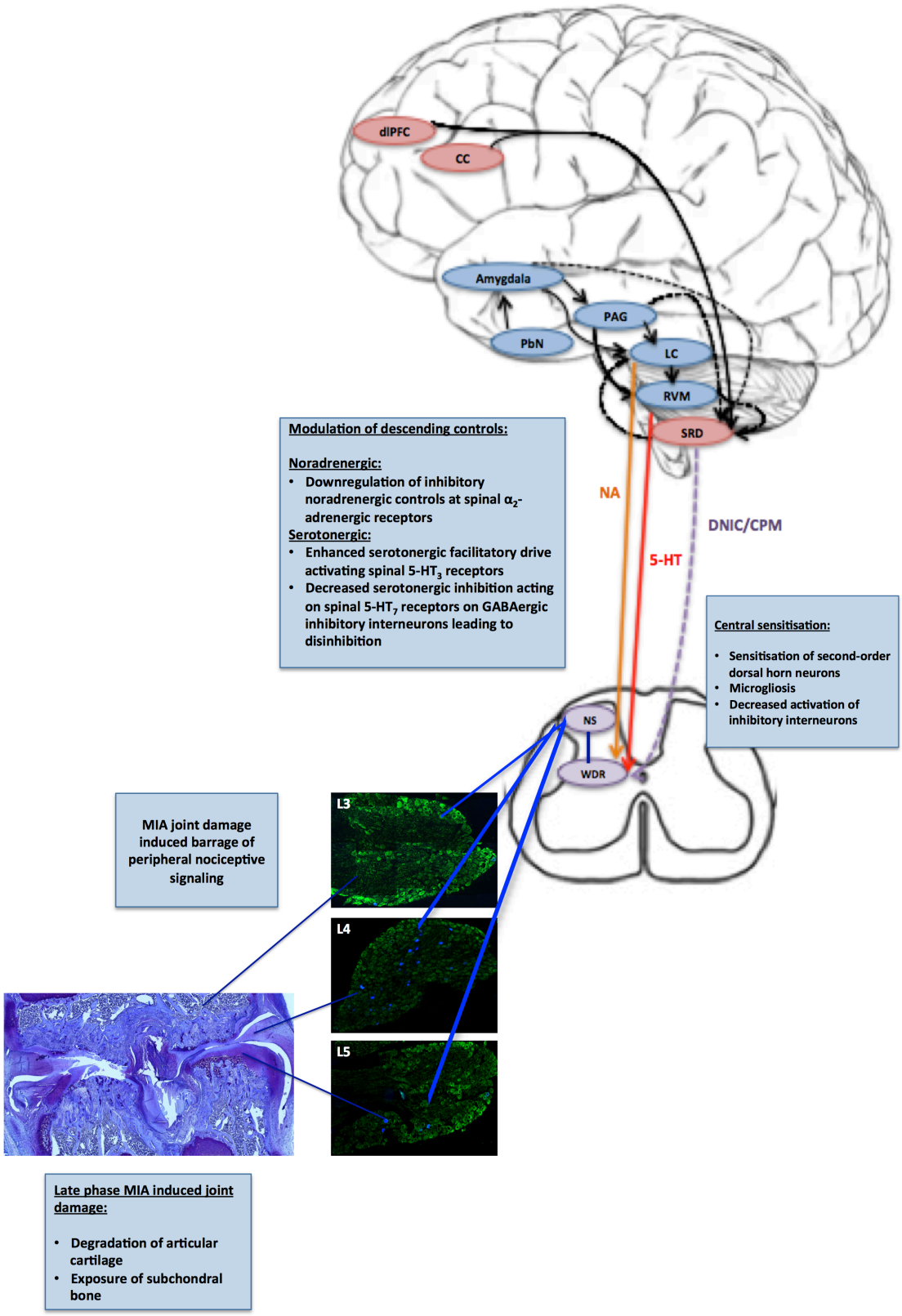


Figure 7.2. The overall schematic of joint damage induced peripheral sensitisation, central sensitisation and modulation of descending controls. The MIA induced joint damage observed in late phase MIA animals results in a continuous barrage of nociceptive signaling from the periphery to the dorsal horn, which can lead to an increased sensitivity of second order neurons and the development of central sensitisation. Subsequently, an imbalance in descending controls develops, such that there is a decreased noradrenergic and serotonergic inhibitory drive acting at spinal α_2-adrenergic and 5-HT_7 receptors, and an increased serotonergic facilitatory

drive acting at spinal 5-HT_3 receptors. The overall consequence is a loss of DNIC, potentially due to altered signaling in the brain structures reported to be involved in the efficacy of DNIC. The brain structures presented in red have been shown to directly impact DNIC, while the brain structures presented in blue are thought to play an indirect role on the expression of DNIC.

8. Appendix

8.1 Pharmacology controls

Pharmacological controls were carried out to ensure any drug effects reported on neuronal firing were due to drug actions rather than the solution they were delivered in. Pre-conditioned neuronal responses and DNIC responses, calculated as a reduction in mechanically evoked neuronal firing induced with a concurrent noxious ear pinch, were tested. A DNIC trial was carried before and after a subcutaneous injection of saline (250µL)(Figure 8.1A), the spinal application of saline (50 µL) (Figure 8.1B), and the spinal application of vehicle (97% normal saline, 2% cremaphor, 1% DMSO)(50 µL)(Figure 8.1C). The saline and vehicle solutions had no effect on pre-conditioned mechanically evoked neuronal firing or on the level of neuronal inhibition induced by DNIC.

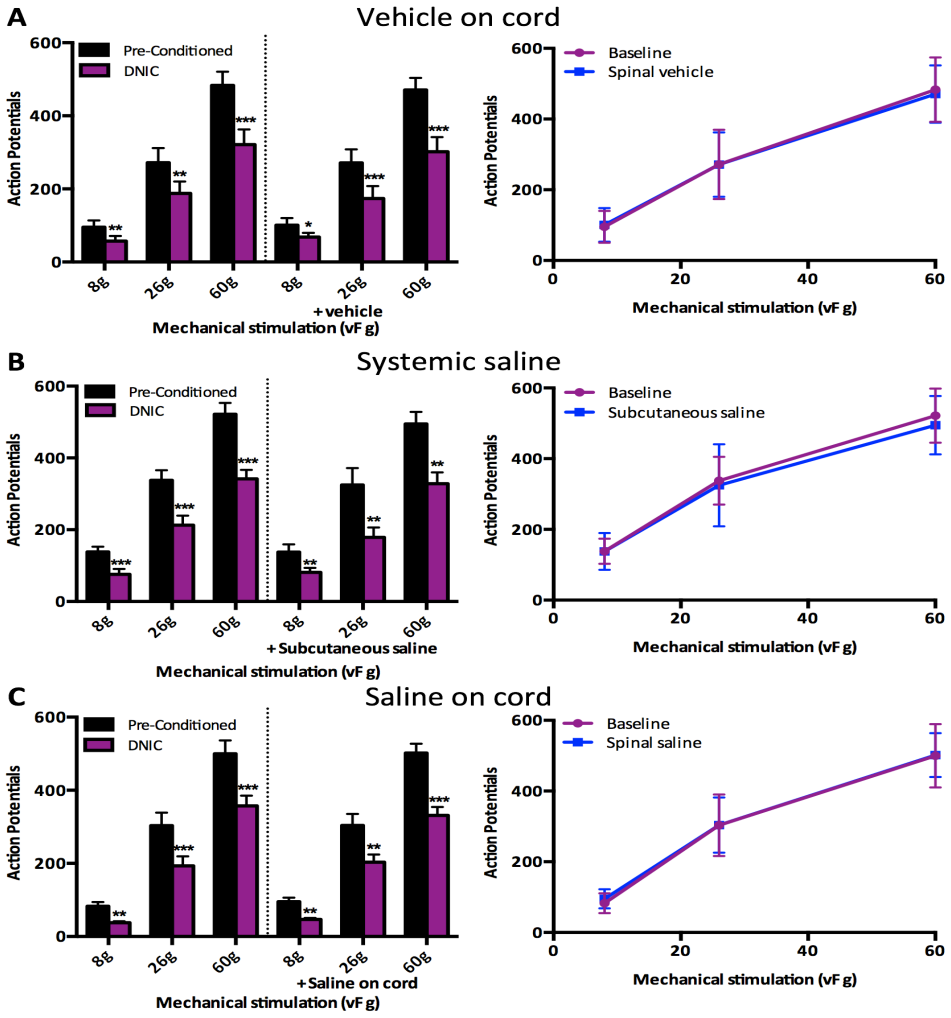


Figure 8.1. Pharmacology controls. A) The topical application of vehicle to the cord had no effect on pre-conditioned mechanically evoked neuronal firing or the neuronal inhibition induced

by DNIC. B) A subcutaneous injection of saline had no effect on pre-conditioned mechanically evoked neuronal firing or the neuronal inhibition induced by DNIC. C) A topical application of saline had no effect on pre-conditioned mechanically evoked neuronal firing or the neuronal inhibition induced by DNIC. Two-way ANOVA with Bonferroni correction. *P<-0.05, **P<0.01, ***P<0.001.

8.2 MIA injected animals and saline injected sham control weights

The MIA injection produced joint damage and pain-like behaviour, but other than that we want the animals to remain in relatively good health. To monitor this we measure the weights of the animals over the course of the model and check the animals are gaining weight in course with saline injected sham controls. No statistical difference was found between the weights of early phase MIA injected and saline injected animals over the course of the model (Figure 8.2A). No statistical difference was found between the weight of late phase MIA injected animals and saline injected sham controls over the course of the model (Figure 8.2B).

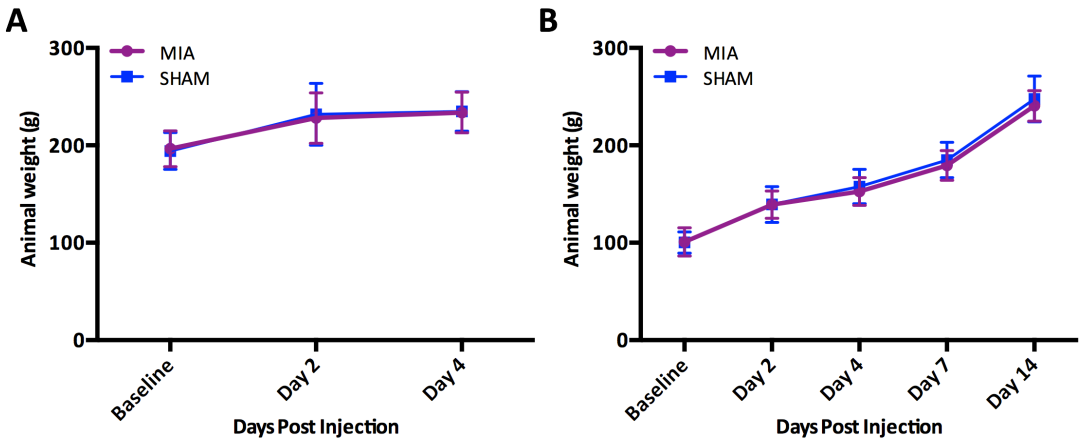
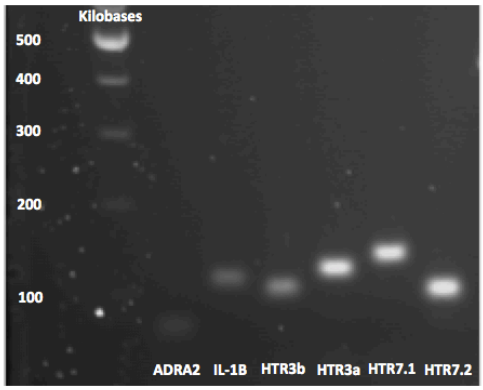


Figure 8.2 The weights on MIA injected and saline injected sham controls over the course of the model. A) There was no difference between the weights of early phase MIA injected animals and saline injected sham controls over the course of the model (EP MIA n=10, EP Sham n=13). B) There was no difference between the weights of late phase MIA injected animals and saline injected sham controls over the course of the model (LP MIA n=8, LP Sham n =10). Two-way ANOVA with Bonferoni correction.

8.3 qPCR primer validation

When new primers were designed using pre-established primer-blast criteria, a serial dilution of each primer was created to produce a standard curve. The resultant PCR product was then ran on an agarose gel using electrophoresis to confirm that only one product of correct length was being amplified. All of the primers used for this thesis that were designed in this way, only one band was produced, and these were all of appropriate length as determined by comparing the band to a DNA ladder (Figure 8.3).



Gene	Product size	Single band
ADRA2	73bp	✓
IL-1B	121bp	✓
HTR3a	109bp	✓
HTR3b	131bp	✓
HTR7.1	146bp	✓
HTR7.2	107bp	✓

Figure 8.3 Primer validations. For all primers designed for this thesis, when the PCR products were ran on an agarose gel only one band was produced of the appropriate size indicating the primers are resulting in the amplification of the correct product.

8.4 Fast Blue neuronal tracer

To confirm that the Fast Blue neuronal tracer was only being taken up by primary afferents innervating the knee the contralateral lumbar DRGs were also taken and checked for unspecific staining. There were no Fast Blue positive cell bodies in the L3-L5 DRGs contralateral to injection (Figure 8.4).

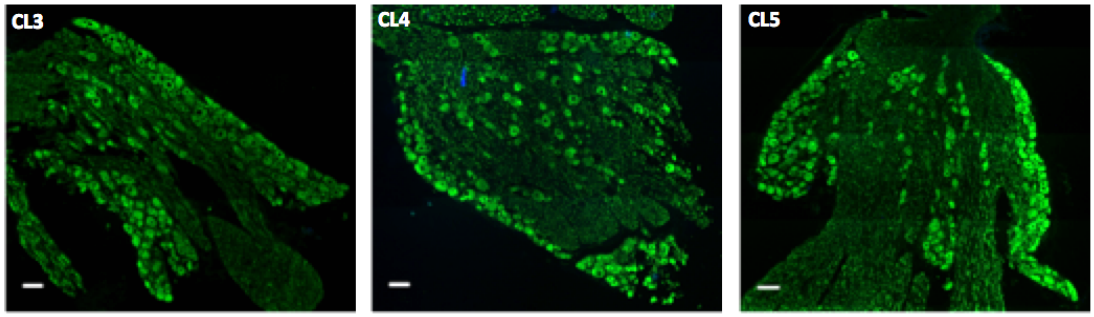


Figure 8.4 Fast Blue staining in the lumbar DRGs contralateral to injection. No Fast Blue positive cell bodies were observed in the L3, L4 or L5 DRGs contralateral to Fast Blue injection.

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